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ON THE MECHANISM OF VENTILATION IN *APHRODITE ACULEATA*

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(With Six Text-figures)

APHRODITE ACULEATA L., the sea mouse, is generally dredged from muddy bottoms, where it burrows head-first into the mud. Usually only the anal extremity, which is very mobile and often held in an upturned position, remains uncovered.

In dorsal view the parapodia are hidden by a beautiful fringe of setae which project from the sides of the body. The true dorsal surface is concealed by a felt formed by long matted threads, which arise from the parapodia. In the cavity enclosed by the felt and dorsal body wall lie the elytra (Fig. 1). These leaf-like structures issue in a double row from the dorsum, which they completely conceal. They are epidermal expansions arising from the dorsal wall of pouches called elytrophores (Fig. 5).

As was described by Fordham (1925, p. 29) the elytra serve to ensure the circulation of water over the dorsum, which is, therefore, considered to be the true respiratory surface. At intervals they are depressed. The depression begins with the anterior pair and passes rapidly backwards. This flattening of the elytra on to the dorsum forces a jet of water out through the exhalent aperture, which is situated at the dorsal side of the anal extremity.

As soon as expiration is completed the exhalent aperture is closed. Immediately afterwards inspiration begins: the elytra are again elevated.

From this point Fordham's description runs as follows (1925, p. 39): "Whilst the water beneath the felt is being rhythmically pumped out by way of the ejection apparatus, *a fresh supply is continually percolating in through the felt*" (my italics).

Hazelhoff (1938, p. 312), however, when

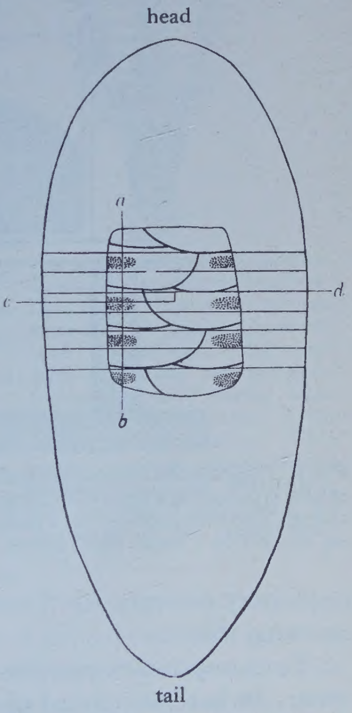

Fig. 1. Diagram of *Aphrodite* in dorsal view. In the felt an opening has been made so that ten elytra are visible. The elytra overlap one another from before, backwards and individually from left to right and right to left, alternately. Six body segments are indicated. It is seen that the elytra, by means of their elytrophores (dotted areas), issue from alternate segments. *a–b*, section represented in Fig. 5; *c–d*, section represented in Fig. 4. Somewhat reduced.

working on the utilization of oxygen in *Aphrodite*, observed a marked current of water going in near the animal's tail. This discrepancy led me to reinvestigate the mechanism of ventilation. The present paper deals with the results obtained.

EXPERIMENTS

The animals used were obtained from the marine Zoological Station at Den Helder. They were apparently healthy; when turned over on their backs they soon righted themselves.

The respiratory currents were made visible by adding, to the ingoing water, a small amount of a Chinese ink suspension prepared by rubbing solid Chinese ink with sea water in a mortar. The fluid thus obtained is very suitable for demonstrating respiratory currents, for it contains no extraneous chemicals and therefore has no irritating effect.

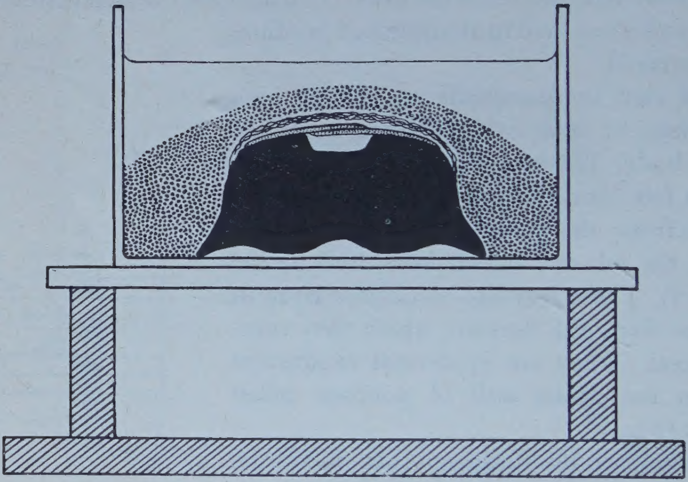

Fig. 2. Diagram of a transverse section of *Aphrodite* passing through a right and left parapodium. The animal is placed in a Petri-dish filled with water and is covered with sand. There is no sand under the animal. The dish is placed on a glass plate, so that the animal's ventral side can be observed with the aid of a mirror. Small dots: elytra. Heavy dots: sand. Matted lines: felt. Somewhat reduced.

First, a specimen was placed in a normal position in a Petri dish filled with sea water. Its head was placed against the wall of the dish, so that it was prevented from moving forward. After some 5–10 min., when the locomotory efforts of the animal had ceased, ink was administered near its tail. In accordance with Hazelhoff's observations, every twenty or thirty seconds a current of water was seen to enter the space under the upturned anal extremity. At the same time another current escaped from the exhalent aperture.

In order to follow the current disappearing under the animal and to approach more closely to natural conditions, I covered a specimen with a layer of sand, about 1 or 2 cm. thick (cf. Fig. 2). The animal soon curved its tail upwards through the surface of the sand. By doing so it gains free access to the water, and is therefore able maintain respiration. Care was taken that there was no sand between the ventral de of the animal and the glass bottom of the Petri dish. The dish was then placed n a glass plate and the animal's ventral side illuminated ith a lamp. In this way the ingoing current could easily e followed with the aid of a mirror. It was found that uring each expiration (i.e. the ejection of water from e exhalent aperture) the animal's ventral side is lifted p from the substratum—as shown in Fig. 4—and ater flows into the space thus formed. The chief paths ong which the water flows are shown in Fig. 3.[1]

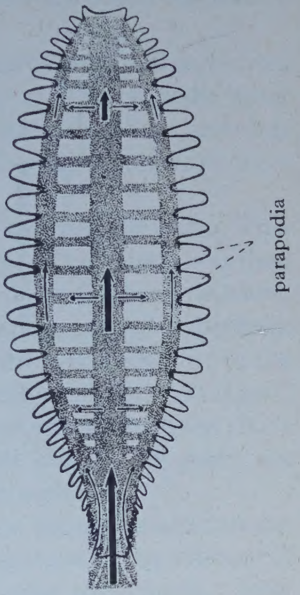

Fig. 3. Main respiratory currents, flowing under a burrowed *Aphrodite* which has only its anal extremity projecting above the sand. Dotted area: water currents. Thin and heavy arrows: currents caused, during expiration, by the elevation of the ventral body surface. Thin arrows: currents caused, during inspiration, by the widening of the respiratory cavity and the depression of the ventral body wall. × ½.

As soon as expiration is completed, i.e. after the xhalent aperture has been closed, the animal's ventral urface begins to flatten again. During this process of attening, the water under the animal, together with ome water from under its tail, is seen to pass between e parapodia. Apparently it is sucked up into the orsal respiratory cavity. That this is actually the case proved by the fact that during the next expiration a tream of ink-coloured water escapes from the exhalent perture.

It thus appears that during expiration the scales ecome more and more depressed.[2] Consequently the ater contained in *C* (Fig. 4*a*) is forced out through the jection apparatus. At the same time the ventral body all is elevated, so that water from under the tail is rawn into the space formed under the body.

During inspiration the scales are again elevated and t the same time the ventral body wall is depressed. In onsequence, water from under the animal passes etween the parapodia and then flows via the inter-egmental openings in the felt (see Fig. 4*a*)[3] into the orsal respiratory cavity *C*.

The same analysis holds good for an animal which is not covered with sand. A pecimen was laid on the surface of the sand, either in a normal position or on its ack. In both cases the ink, administered between some of the parapodia, was eadily sucked up at the next inspiration. At the next expiration it re-appeared in he outgoing current.

On the other hand, when some ink was pipetted on to the dorsal felt, no suction hrough the latter could be observed through a binocular microscope.

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[1] The ventral body surface, between the segments, shows more or less well-defined grooves. It is articularly in these grooves that the water is seen to flow in a lateral direction.

[2] In an animal covered with sand, the dorsal felt follows the movements of the scales very closely. n specimens lying free on the substratum the felt seems to move to a smaller extent.

[3] The felt is formed by threads which issue from the parapodia, and exhibits small lateral inter-egmental openings.

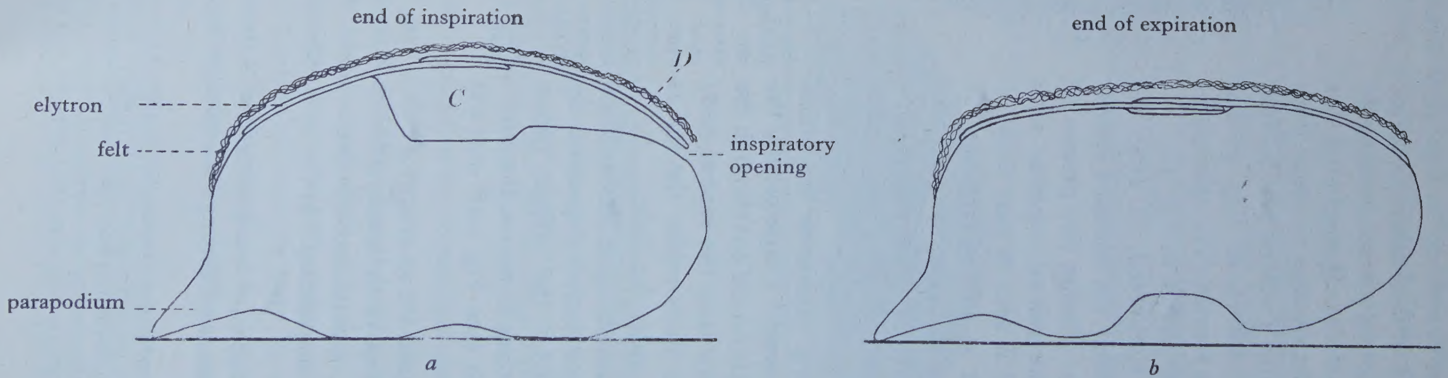

Fig. 4. Diagrams of transverse sections through *Aphrodite* showing the respiratory movements of dorsum, scales, etc. The degree of movement was somewhat roughly estimated. Each section passes on the left side through a parapodium, on the right side between two parapodia (*c–d*, Fig. 1). ×2½.

From the above, it is clear that Fordham's view must be rejected. The inhalent urrent does not percolate continuously through the felt, but passes, during inspiraon only, through the intersegmental openings in the felt into the dorsal respiratory vity.[1]

The lateral apertures in the felt open both into the space between felt and elytra), Fig. 4) and into that between elytra and dorsum (*C*, Fig. 4). Therefore the water ight flow directly into *C* or first enter *D*, then pass between the scales and thus ow into *C*. To decide between these alternatives the following experiments were rried out.

An opening was made in the felt, thus exposing one or two pairs of elytra. This pening was covered with a piece of transparent cellophane. The animal was then ntirely covered with sand, except over the centre of the cellophane window. hereupon, ink was added to the ingoing current, either under the tail or between ose parapodia which were situated at the left or right side of the window; in the tter case, of course, these parapodia were not covered with sand. In this experient, no ink was seen to stream over the upper side of the elytra, i.e. between elytra nd cellophane, although the exhalent current was distinctly black. Apparently the spiratory current flows directly into the space under the scales.

The following experiments with methylene blue confirm the above conclusions. specimen was covered with sand, and with the aid of a pipette a strong solution of ethylene blue was added to the ingoing current for some 30 minutes. After moval of the felt, all parts of the body surface and elytra were found to have been ained blue, except (1) the upper sides of the elytra and (2) those parts of their wer sides which were covered by a part of the adjacent elytra (cf. Fig. 5).

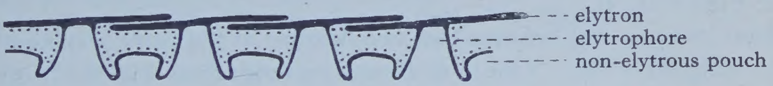

Fig. 5. Diagram of a longitudinal section through scales and elytrophores (*a–b*, Fig. 1). Dotted: surface coloured by methylene blue (see text). × 2.

A specimen, not covered with sand, was left for some 45 min. in a strong lution of methylene blue. The felt was then removed. Again, only the exposed wer sides of the elytra had been coloured. If an animal was left for a longer time this solution, the inner side of the felt and the upper sides of the elytra were also und to be stained, but at first only at those places where the felt was rather thin. pparently, in this case, some dye had permeated through the felt. It seems quite ear that the respiratory current does not enter the space between felt and elytra, ut flows directly into that under the scales.

In some text-books, it is stated that the respiratory current flows "between orsum and felt" (Buddenbrock, 1928, p. 309) or flows "beneath the felt" (Hempelann, 1934, p. 127). It is clear that these statements are incorrect, for beneath the felt, i.e. between felt and elytra, no respiratory current is found. For the same reason the statement of Nierstrasz & Hirsch (1922, p. 41) that the felt covers the respiratory cavity, is incorrect.

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[1] Nierstrasz & Hirsch (1922, p. 41) described an anterior, inspiratory opening in the felt. This is roneous; such an opening is only present in damaged specimens.

In *Hermione*, the dorsal felt is absent. In this genus the inspiratory openings are also situated laterally between the body and the scales (Claparède, quoted by Pagenstecher, 1875). Therefore, the mechanism of ventilation in *Aphrodite* and *Hermione* may be assumed to be of essentially the same nature.

We may now consider how the animal, during expiration, prevents water from flowing back through the inspiratory openings in the felt.

To solve this question, I made an opening in the felt and removed parts of the scales, thus exposing some elytrophores and non-elytrous pouches. By lifting up these scales somewhat and looking under them in a lateral direction it is possible to observe the true inhalent apertures (cf. Fig. 4*a*). The lateral walls of these openings are formed by the dorsal side of an elytrous and a non-elytrous pouch respectively whilst the roof is formed by an elytron.

During expiration, as already shown, the ventral body wall is drawn concavely inwards. Moreover, elytrous and non-elytrous pouches are pressed against one another so that the narrow channels between these (cf. Fig. 5) are closed. At the same time both dorsum and non-elytrous pouches are elevated (cf. Fig. 4)[1] and the scales depressed. In other words: during expiration elytra and dorsum move towards one another. During the first stage of expiration, the inspiratory openings are closed; at a later stage elytron and non-elytrous pouch are pressed against one another over a considerable area (cf. Fig. 4*b*). In this way, water in the respiratory cavity is prevented from flowing back through the inspiratory openings; it does not escape between the elytra, since these structures overlap one another over a considerable length (cf. Figs. 1, 4, 5).

Fordham (1925, p. 39) believes that two flexures of the body play an important part in elevating the scales: "One is an antero-posterior flexure of the trunk . . . with its concavity ventralwards. The other flexure is at right angles to this; its concavity is also ventralward, by it the right and left margins of the body are drawn slightly together below." Such flexures may be observed, especially those of the latter type. As far as I could see, they are less marked than is indicated in Fordham's drawing (1925, p. 40) and occur during expiration only, i.e. during the depression of the scales. In my opinion, depression of the scales is caused by a muscular contraction of the elytrophores, whereas the elevation is brought about mechanically by the pressure of the coelomic fluid. The cavity in the elytrophores is in open communication with the coelomic cavity.

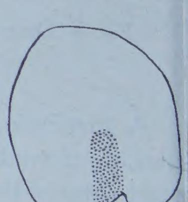

Fig. 6. Dorsal surface view of a right elytron. The area of attachment is dotted. × 1½.

Fordham's drawing (1925, Fig. 7*b*, p. 40), is incorrect in two points. First, because it indicates that the right and left margins of the body, i.e. the tips of the parapodia, which lie on the substratum, display vertical movements. Secondly, because in a transverse section of the animal passing through an elytrophore, the scales in reality do not show a free lateral area (cf. my Figs. 4 and 6); for this reason Fordham's Fig. 38, Pl. V, is also incorrect.

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[1] According to Fordham (1925, p. 39) the first sign of expiration is an elevation of the elytra to their maximum extent. In my opinion this "elevation" is probably brought about, not by the extension of the elytrophores but by the elevation of the dorsum, which may occur somewhat prior to the depression of the scales.

SUMMARY

In *Aphrodite aculeata* the floor of the respiratory cavity is formed by the dorsal body wall, the roof is formed by the elytra. Ventilation is effected by pumping movements both of the dorsum and of the elytra.

During inspiration water is sucked into the respiratory cavity via lateral inspiratory openings in the felt.

During expiration the inspiratory openings are closed, and dorsum and elytra move towards each other. Consequently, the water in the respiratory cavity is forced out through the exhalent aperture, situated at the tip of the anal extremity.

When burrowing, the animal holds its tail in an upturned position above the sand. It has free access to the water *via* the space formed, during expiration, between ventral body surface and sand.

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THE REFLEX MECHANISM OF THE INSECT LEG

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(With Four Text-figures)

INTRODUCTION

In attempts to resolve animal behaviour into simpler terms much weight has always been given to the concept of the reflex as a unit. Since the classical work of Sherrington and others (Creed, etc., 1932) on the responses of the spinal vertebrate, many authors have supposed it to be merely a matter of time before all behaviour would be interpreted in such terms. In recent years, however, another school of thought seems to be gaining ground; in the works of Coghill (1929) and v. Holst (1936) the idea of patterns of behaviour, inherent in the nervous system and influenced only to a limited degree by external events, is given increasing prominence, while the reflex is relegated to second place. Gray & Lissmann (1938 *a*, *b*), in studies of the locomotion of the earthworm and leech, discuss at some length the relative merits of the two concepts and come to the conclusion that neither can be excluded from the general picture, though one may be more important than the other in special cases. It is the purpose of this paper to enquire how far reflexes in the true sense of the word are present in the make-up of a running insect (cockroach).

REVIEW

Previous evidence for the existence of reflexes in the insect leg may be divided into subjective and objective classes. Many authors have found it necessary to postulate reflex mechanisms in order to explain their observations of the grosser aspects of insect behaviour, but few have produced any objective evidence for their existence. Of the former only those which have a direct bearing on the present work will be considered here.

Hoffmann (1933) described a righting reflex in the cockroach which was initiated by absence of contact of the legs with the ground. Although he did not manage to decide between tactile and pressure stimuli as the normal means of inhibiting this reflex, he claimed that the fact that the pawing movements are immediately stopped in any leg that comes in contact with a rigid object is evidence that there must be some local response system for each leg, in fact what may be called a reflex.

Fraenkel (1932) and v. Holst (1935) describe more elaborate effects of contact of the legs with the ground on the regulation of flight and walking movements spectively. Fraenkel showed that the movements of flight are inhibited immediately y contact stimuli to the legs, while v. Holst maintained that it is the proprioceptive fluences from the legs that regulate the general co-ordination. But the most nportant work, where the assumption of local responses becomes essential for the ypothesis put forward, is that of Crozier & Stier (1927–8, 1928–9) on geotropic ientation. These authors showed that, in spite of the absence of any special avity-sensitive organ, insects are capable of accurate geotropic orientation, and ostulated instead elaborate proprioceptive reflexes, working as they thought from nse organs in the muscles, and enabling the force exerted by each leg to be related irectly to the proportion of the body weight borne by that leg. No actual proof the existence of such reflexes was, however, given.

Actual evidence of the presence of reflexes in the leg system was provided by e work of Rijland (1932 *a*, *b*), who recorded electrical "impulses" in the leg muscles f a number of insects and showed that their frequency was influenced by moveent of the joints, and also by changes in the position of the centre of gravity of e body during respiratory movements. His results will be more fully discussed uring the course of the paper in the light of the observations to be described.

EXPERIMENTAL METHODS

The electrical changes in the muscles of the legs of the cockroach (*Periplaneta nericana* L.) have been recorded with the aid of a condenser-coupled amplifier nd Matthews oscillograph. As was shown in a previous paper (Pringle, 1939), most f the muscles of the cockroach leg are innervated by only two nerve fibres, impulses om one producing a small electrical effect in the muscle and a tonic contraction, hose from the other a larger electrical effect and a rapid twitch contraction. From e oscillograph records from the muscles it is easy to distinguish the two types of lectrical effect, and the frequency of the spikes may be taken as an indication of the ate of excitation of a single unit in the central nervous system.

For the experiments the cockroach is held on its back on a paraffin block by ins through the sides of the thoracic tergites. When intact insects were used the egs other than the one under study (usually one of the metathoracic legs) were llowed to make contact with a strip of paper across the body. Unless this is done he animal makes periodic attempts to right itself and these upset the results. or experiments on the isolated metathoracic ganglion the connectives were cut front and behind with the aid of a pair of scissors, and in some experiments the hole of the ganglion was exposed for electrical stimulation of the nerves.

The main coxal extensor trochanteris and the extensor tibiae muscles have been sed respectively as examples of depressors and levators. The former is innervated rom the large, the latter from the most anterior of the small nerves to the leg. ach has one "quick" and one "slow" nerve fibre (Pringle, 1939). The variation the frequency of the slow fibre discharge and the incidence of bursts of impulses the quick fibre were followed under various types and intensities of sensory timulation.

RESULTS

The depressor reflex

In the intact preparation a record from the extensor trochanteris muscle (depressor) of the metathoracic leg usually shows a steady rhythm in the slow fibre at about 5–20 per sec., even when the leg is free. If the contraction of the depressor muscles is resisted by contact of any part of the leg with a solid object, an increase in frequency is to be observed. Rijland (1932*b*), in the course of a study of this effect in *Hydrophilus*, concluded that forced movements of flexion or extension were necessary to produce the reflex, and that a lasting increase in frequency in response to flexion occurred only in the isolated ganglion preparation, the intact animal becoming "adapted" to the new position. His results did not seem to show exactly what were the conditions for the reflex response.

A number of experiments were therefore performed to elucidate more clearly which sense organs in the leg were responsible for the effect, and exactly what relation held between the movement or position of the limb and the frequency of the tonic discharge. The results are given below in summary form.

(1) Flexion at either the coxo-trochanteral, femoro-tibial or tibio-tarsal joints produces an increase in frequency, as also does any bending of the leg at the trochantero-femoral hinge. In order to simplify the problem the leg was therefore amputated at the distal end of the femur, and §§ (2) and (3) below refer to such a preparation.

(2) Fig. 1 A, B, show records taken during a sudden decrease and increase respectively of the resistance to extension of the femur. The femur was moved by a metal rod attached to a solenoid and wired in series with the marker on the record in order to give the exact moment of the stimulus. In this way while movement of the rod in one direction flexed the femur and increased the tension on the extensor trochanteris muscle, movement the other way merely released the tension and did not forcibly extend the leg, which took up its new position gradually as the muscle relaxed. Results of similar experiments are shown graphically in Fig. 2A, B, C.

In general, a sudden reduction in the resistance to extension causes the motor impulses to cease momentarily and then restart at a lower frequency; with a sudden increase there is a short high-frequency burst, sometimes followed by a "silent period", and then the discharge continues at a higher level than before. With slower changes in pressure the frequency changes gradually to its new level; only over a very long period is there any "adaptation" to the new conditions.

(3) Experiments were performed to determine the nature of the sense organs responsible for the reflex. Three possibilities were considered:

(A) *Internal endings* in the coxa—muscle endings or chordotonal organs.

(B) *Position receptors*—the trochanteral hair plate (Pringle, 1938*b*).

(C) *Stress receptors*—the trochanteral groups of campaniform sensilla (Pringle, 1938*a*).

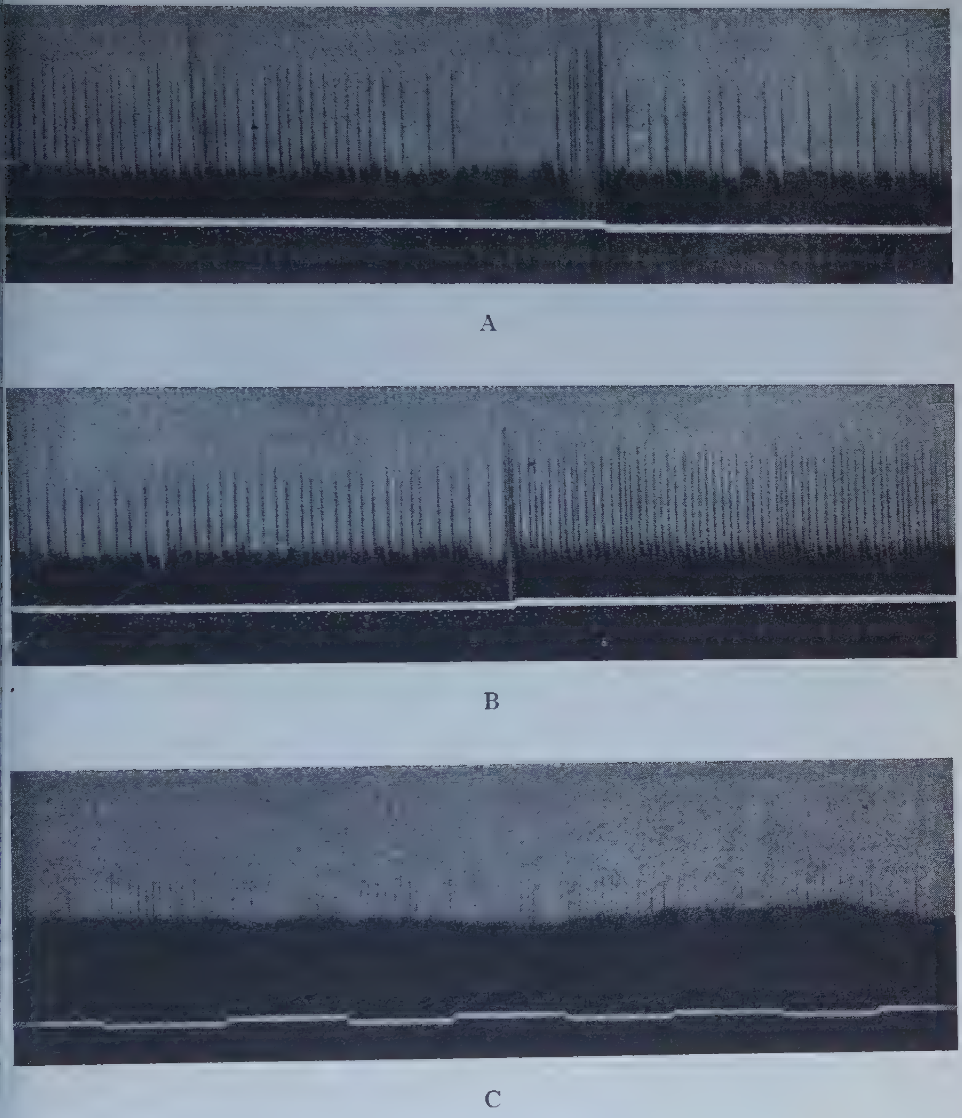


Fig. 1. Records from the extensor trochanteris muscle of the metathoracic leg. A, B. Variations in the slow fibre frequency in response to (A) a sudden decrease, (B) a sudden increase in the resistance to depression of the same leg. C. Slow fibre discharge during passive movement of the leg of the opposite side. Records read from right to left; time marker $\frac{1}{10}$ sec. The stimulus is indicated by the shift in the continuous line; in (C) "up" indicates flexion, "down" extension.

The results are summarized below:

(*a*) Cautery of the nerves in the trochanter stops the reflex. After this treatment the muscle could still be excited normally by other types of reflex stimuli from the body and endings in the coxa should not have been harmed.

(*b*) The apodeme of the extensor muscle was cut and attached to a thread. Tension on this muscle, provided the rest of the leg did not move, had no effect on the frequency of the discharge.

These two experiments eliminate (A) above.

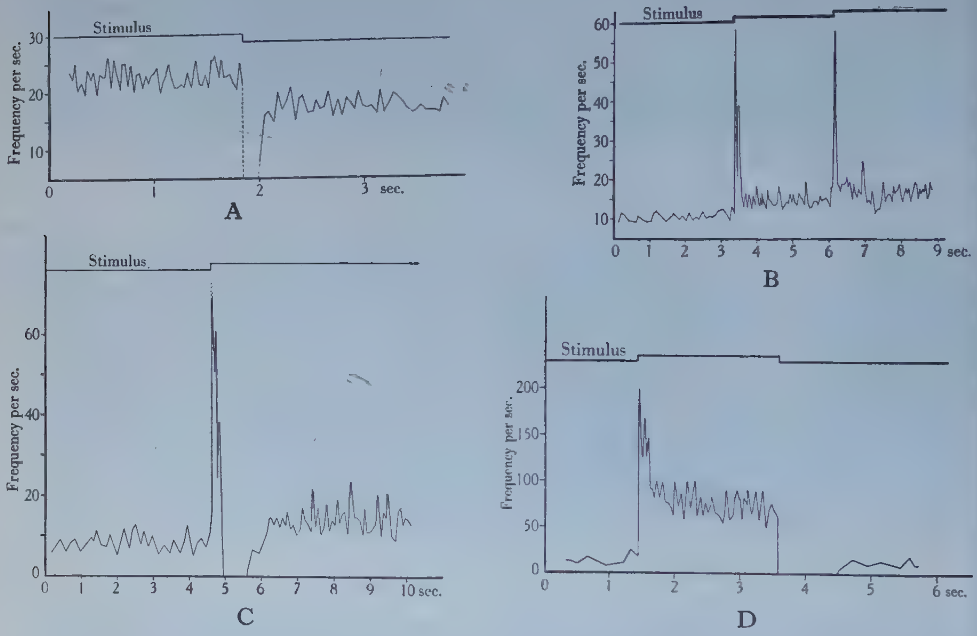


Fig. 2. Graphs of the variation of the slow fibre discharge to the metathoracic extensor trochanteris muscle. A. Reduction in resistance to depression. B, C. Two different results of a sudden increase in resistance to depression. D. Pressure on the trochanter. The frequency at any given moment is plotted as the reciprocal of the interval since the last impulse.

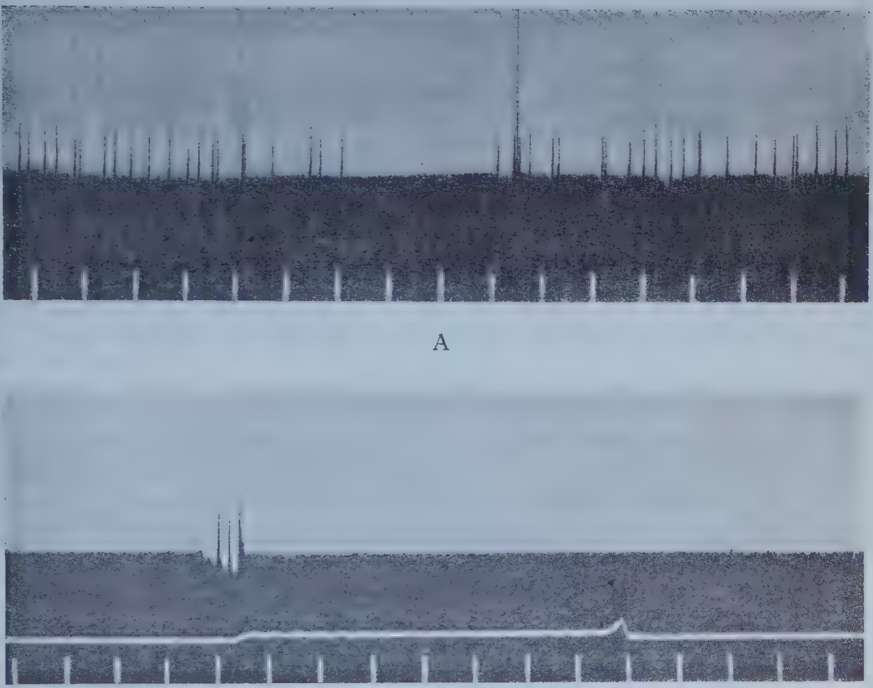
(*c*) It can be seen in this preparation that the frequency of the discharge is quite independent of the initial position of the leg. Moreover, in the experiments described above it is to be noted that the cessation of the motor discharge occurs immediately on removing the resistance to extension of the femur although the femur does not instantaneously take up its new position owing to the muscle viscosity. There is thus no real correlation between the changes in frequency of the discharge and the actual position of the joint.

(*d*) It was noted by Pringle (1938*a*) that the most intense excitation of the campaniform sensilla could be obtained by pressure of the cuticle of the segment in which they lie. Pressure on the cuticle of the trochanter has a profound influence on the frequency of the motor discharge to the extensor muscle, modifying it in the same way as movement of the leg (Fig. 2D). With strong steady stimuli the frequency of the reflex discharge declines at a rate which corresponds closely with the adaptation rate of these campaniform sensilla.

This response to pressure can be obtained when there is no movement of the trochanter on the coxa and also when the apodeme of the extensor muscle is cut.

The conclusion is therefore reached that it is the campaniform sensilla on the rochanter that are responsible for the reflex.

(4) As was also pointed out by Pringle (1938 *a*), most of the campaniform sensilla on the leg of the cockroach are arranged in such a way as to be excited when the nsect is standing on the ground in the normal position. The evidence that these ense organs are responsible for the reflex influence on the tone of the depressor muscles of the leg completes the objective proof of the existence in the insect of proprioceptive mechanism analogous to that of vertebrates.



ig. 3. A. Isolated metathoracic ganglion preparation. Record of slow fibre impulses to extensor ibiae muscle. Cessation of discharge in response to an electrical stimulus to the central end of the ensory nerve of the same side. B. Intact insect. Rebound excitatory burst of impulses in quick bre to extensor tibiae muscle on removal of pressure stimulus to the trochanter of the same leg. Low gain record. Records read from right to left; time marker $\frac{1}{10}$ sec.

Levator reflexes

Under this heading may be considered two reflex effects that have been observed n the extensor tibiae and other levator muscles.

1) *Muscle antagonism and depressor rebound.*

Records from the levator muscles during stimulation of the campaniform sensilla how a variation in the frequency of the slow fibre impulses that bears a close nverse relation to that in the depressor set (cp. Rijland, 1932 *b*). Fig. 3 A shows the effect on the frequency of the slow fibre impulses to the extensor tibiae of short duration electric shocks applied to the central end of the large leg nerve of the same side. This nerve contains other fibres besides those from the campaniform sensilla, but the reflex inhibitory effect is well shown (isolated ganglion preparation).

With light stimulation of the campaniform sensilla by pressure on the trochanter the extensor tibiae slow fibre discharge may merely be reduced in frequency for the duration of the stimulus, returning thereafter to its normal level, but after strong stimulation there is a marked rebound, which may affect also the quick fibre. Fig. 3 B shows this result in a preparation where there was no resting discharge. This rebound corresponds with the silent period in the depressor reflex and may play an important role in normal locomotion (see below).

(2) *Response to touching the leg.*

Tactile stimuli when applied to the upper surface of the tarsus or to the tibial spines lead to a momentary lifting of the leg so that it is placed on top of the stimulating object. With light stimuli only the slow fibres of the levator system are involved; stronger stimuli bring in the quick fibre also and may initiate the rhythmic pawing movements of the "Suchenreflex" (Hoffmann, 1933), particularly in the decapitated animal. In either case excitation of the levator fibre is accompanied by a corresponding inhibition of the depressor sets.

The antagonism of the depressor and levator systems is well brought out by the balance that can be struck between them. Simultaneous stimulation of sense organs on the leg tending to elicit opposite responses may cancel out. The most usual case of this is when the leg is pressing against a solid object with one of the backwardly directed tibial spines. Stimulation of these spines alone elicits the levator response, but the force of resistance to depression of the leg also excites the campaniform sensilla of the trochanter and the balance is held on the depressor side. Further excitation of the spines can ultimately produce the levator response in spite of the simultaneous excitation of the campaniform sensilla (cp. the results of Morson & Phillips (1936) on the reflex antagonism between the A and B endings in the cat vasto-crureus muscle).

Crossed and intersegmental effects

There is a slight but definite effect of stimulation of the campaniform sensilla of one side, on the frequency of the slow fibre impulses of the other side. Fig. 1 C shows a record from the right metathoracic extensor trochanteris (depressor) while the leg on the opposite side was moved passively with rhythmic extension and flexion. The frequency is regularly reduced during the forced flexion movements.

In the isolated ganglion preparation electrical stimulation of the central end of the large sensory nerve while recording from the extensor tibiae of the other side produces a slight increase in the frequency of the discharge, followed always after an interval of about half a second by a silent period. This may be the accompaniment in the levator muscles of a rebound excitatory burst in the depressors.

In contrast to these results is the complete failure of many attempts to show eflex effects transmitted along the cord from one leg to another. In the intact reparation, handling or electrical stimulation of any part of the body produces temporary rise in both the depressor and levator discharges, but no regular effect n the depressor-levator balance of one leg could be observed in response to any orm of stimulation of the sense organs of the legs of another segment. The three airs of thoracic legs appear to be completely independent as far as their reflex ystems are concerned.

Influences from the body

As has been stated above, any stimulation of the body of the insect tends to ncrease the frequency of both the depressor and levator fibres. Some types of timuli, however, have more specific effects.

Touch on the side of the abdomen decreases the discharge to the depressor uscles of the same side and increases those of the opposite side. This would have he effect in the normal standing position of moving the abdomen away from the timulus.

A marked effect is produced by draughts of air on the anal cerci (cp. Pumphrey Rawdon-Smith, 1937). Each puff of air excites the depressor muscles of all the egs and evokes also a burst of impulses in the quick fibres, producing a co-ordinated ump forwards.

The effect of section of the connectives

Section of the connectives in different places has very constant effects on the eflex behaviour of the preparation.

Removal of the head produces a state of hyperactivity of all the slow fibres, ut particularly the depressors. The animal stands in a position reminiscent of ecerebrate rigidity in the mammal. The leg reflexes are all present but levator esponses tend to merge very readily into states of disco-ordinated activity in which ll the legs are moved violently to and fro. The response to draughts is increased nd the insect appears generally to be in a hypersensitive condition.

Isolation of the ganglia (experiments have mostly been done on the metathoracic anglion) has a very different effect, the general tone being much lowered and the mphasis now rather on the levator sets. Some such preparations will show a slow esting discharge to the levator muscles and none to the depressors; others have o tonic activity at all. Nevertheless the reflex effects are still present and normal, nd the preparation appears merely to be somewhat inexcitable.

DISCUSSION

Two points in particular arising out of the foregoing account seem to need urther discussion; namely, the question of the excitation of the quick and slow ibres, and the origin of rhythmic movements.

In general it seems that the quick fibre is called into action by the same types f reflex stimuli that excite the slow fibre, but that it requires a greater intensity f excitation. In particular the quick fibre seems to be involved in rebound phenomena and rhythmic movements, while the slow fibre responds to prolonged reflex stimulation. There are no facts that would not fit a scheme in which the two fibres are supposed to have the same central connexions, but a difference in threshold and accommodation. A sudden rise and fall of central excitation will then excite both fibres, while a slower increase will normally bring in only the slow. Observations on peripheral nerves have shown that fibres with the greatest capacity for accommodation are also most liable to be excited by "off" effects, and this may explain the sensitivity of the quick fibre to rebound. Otherwise it is most easily called into action through the "pseudo-giant fibre" system from the anal cerci (Pumphrey & Rawdon-Smith, 1937); possibly the large size of these fibres causes a large and sudden rise in central excitation on the arrival of each impulse at the motor centre.

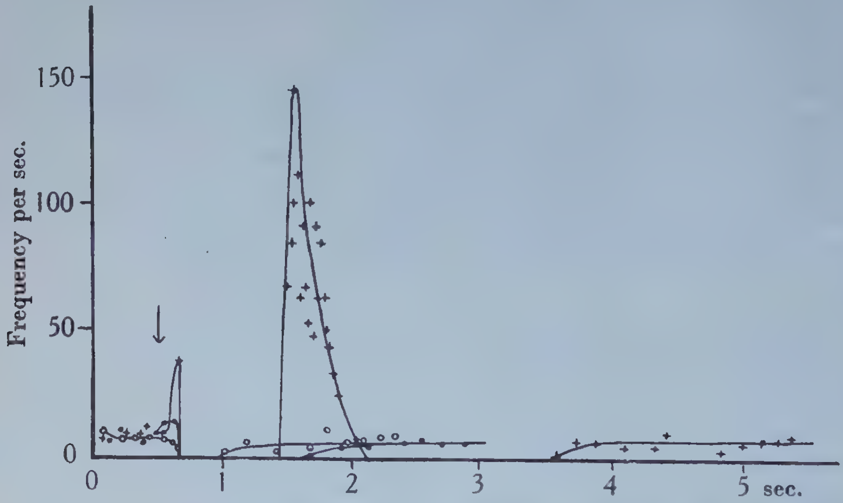


Fig. 4. Graphical representation of variations in the frequency of slow fibre impulses to the metathoracic extensor trochanteris muscle in response to different intensities of stimulation of a single tibial spine of the same leg. Smooth curves have been drawn through the three sets of points in order to present a clearer picture of the changes in central excitation.

On the question of the nature and origin of the rhythmic movements that play such an important part in normal locomotion, it is by no means so easy to speculate. In some of the preparations studied there have been signs of rhythm in a sort of double or treble rebound after the cessation of sensory stimulation, particularly in the decapitated animal after the levator response to touching the leg. Fig. 4 shows the result of one experiment where an attempt was made to determine accurately the conditions for this phenomenon. The preparation showed a steady slow fibre discharge to the depressor muscles, and this could be momentarily inhibited by movement of a single tibial spine (levator reflex as described above). By gradually increasing the intensity of sensory stimulation it was possible to reach a point where the inhibition, instead of being followed by a rapid return to the normal frequency, was followed by a short high-frequency burst, involving also the quick fibre, followed by another silent period and then a return to normal.

A still higher intensity of stimulation would produce several of these alternating bursts and silent periods before the insect again quietened down. It was as though each excitatory burst, alternatively to the depressor and levator systems, stimulated the next to activity, but that the rebound was not quite sufficiently strong to keep up the rhythm for more than a certain period against a natural damping effect. The stronger the initial stimulus, the longer could this rhythm persist. Possibly in the normal walking movements sensory stimuli are continually arriving at the motor centres in sufficient intensity to maintain the alternation.

It should be noted in this connexion that rhythmic movements of the legs do occur even when they are out of contact with the ground ("Suchenreflex" of Hoffmann, 1933). Under these conditions there is no stimulation of tactile or campaniform sensilla and the rhythm must either be spontaneous or generated by reflexes other than those described here, possibly from the hair plate or chordotonal sensilla. Such a state of activity could, however, be produced in each of the leg systems by an increase in the central rebound phenomena. Further elucidation of the nature of this effect and of its control by higher centres may give the clue to the origin of spontaneous movements.

SUMMARY

1. Variations in the frequency of the motor discharges to the leg muscles of *Periplaneta americana* are followed in the intact animal under different types of sensory stimulation by electrical recording from the muscles.

2. Two main reflexes are described: the depressor reflex, evoked by stimulation of the campaniform sensilla on the legs, and a levator response to touch on the upper side of the leg.

3. There is a direct antagonism in the excitation of the depressor and levator sets of muscles: also between similar muscles in the two legs of a segment.

4. Reflex effects are not transmitted up or down the animal to other segments.

5. The conditions for the reflex excitation of the two types of motor fibre are discussed.

6. A suggestion is put forward for the explanation of rhythmic movements.

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UTILIZATION AND DIGESTION OF CARBOHYDRATES BY THE ADULT BLOWFLY

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It has been frequently stated that many species of flies, including houseflies and blowflies, can be kept alive and healthy on a diet consisting only of cane sugar and water; on this they are able to live for the maximum length of time, which, in the case of the blowfly *Calliphora erythrocephala*, is about 2 months at 27° C. Addition of protein is essential only for the development of the eggs, as it does not increase the duration of life and there is, indeed, evidence that it may have the contrary effect. Flies thus represent an excellent medium for studying problems of carbohydrate metabolism, and the present investigation deals particularly with two questions: what effect have substances other than cane sugar on the length of life of the fly, and to what extent does the presence and absence of enzymes in the gut account for the utilization or non-utilization of these various substances?

The first part of the investigation consisted of a series of feeding experiments with different sugars and allied substances and, in extent, was necessarily limited by the extreme rarity and scarcity of many of the substances concerned, the quantities available being in some cases very small (1–2 g.). A preliminary account of the feeding experiments has already been given (Fraenkel, 1936).

FEEDING EXPERIMENTS

In all the experiments the flies were fed from the time of emergence on a diet which consisted solely of water and the substance to be tested. The flies were kept in 7 lb. jam jars, which had a filter paper overlying a layer of sand at the bottom to keep conditions as dry and clean as possible. Twenty flies were normally used in each experiment, but when the amount of available diet was limited the number was reduced to fifteen or ten. The temperature was kept constant at 27° C. throughout. Most substances were fed dry and in quantities which lasted for only a few days so as to minimize contamination, water being supplied by means of soaked cotton-wool. In all cases where the length of life was not appreciably increased by the special diet when given in dry form the experiment was repeated with the substance dissolved in water, the soaked cotton-wool being then omitted. This was to exclude the possibility that the special diet might be tasteless, or even possess an unpleasant taste, and thus be refused by the flies, which would be forced to take it, however, when thirsty, in the repeat experiment. Such solutions had to be changed daily to obviate contamination, so that much larger quantities of material were required than was the case when the diet was fed dry. For this reason some of the rarer substances could not be fed in liquid form. Fortunately, in almost every case wherein a substance proved to be of great nutritional value it was eaten in the dry state as readily as cane sugar itself, and the only substances, namely, lactose, starch and glycogen, on which the flies subsisted for an appreciably longer period when fed in aqueous solution, were available in quantity.

A fly deprived of water and all nutrient lives for about 2½ days, while that given water alone may live up to 3½ days. Every increase in length of life above about 4 days therefore can be ascribed to the positive effect of the added nutrient. In most experiments it was found that one or two flies will outlive the rest for a considerable period, as will be seen from the following experiment, which is typical.

Twenty flies on a diet of α-methyl-glucoside

Up to 26th day	First 10 flies were dead
On 28th day	11th and 12th flies were dead
On 33rd day	13th and 14th flies were dead
On 34th day	15th and 16th flies were dead
On 36th day	17th fly was dead
On 40th day	18th fly was dead
On 49th day	19th fly was dead
On 56th day	20th fly was dead

It seems, therefore, that the number of days which the longest living fly in any one experiment lives does not give a good indication for the nutritional value of a particular diet concerned, and a much more significant average figure for survival was considered to be the number of days before half the total number of flies were dead. This figure is also much less liable to be influenced by the number of flies used in the experiment.

The results of all the feeding experiments are given in Table I. They show that the carbohydrates used can be divided roughly into three groups: (1) substances on which flies survive well, half the insects being dead in about 18–35 days, (2) substances on which survival is no better than on water alone, i.e. about 3–4 days, and (3) substances on which the flies survive for a few days longer than those insects receiving water alone.

Group 1. The first group consists of the hexoses glucose, fructose, galactose and mannose; the disaccharides sucrose, maltose, trehalose, melibiose; the trisaccharides raffinose, melezitose; the polysaccharide dextrin; the glycosides α-methyl-glucoside, α-methyl-galactoside; and the sugar alcohols mannitol and sorbitol, for the flies live on these substances quite as well as on sucrose. The difference in the number of days which the flies lived on these substances may have some significance, but it is not regarded as a valid one in these experiments, for one always finds variations in the viability of flies which are reared at different times and in different batches. The viability may be influenced, for instance, by the amount of food eaten by the larva, the temperature at which the larvae were reared and also the particular conditions of the experiment, such as humidity and infection with micro-organisms. To obtain differences which could be regarded as significant would require more standardized conditions of breeding, the use of pure strains, the employment of a much greater number of flies and several replicates of each experiment. In most cases the amount of material available as diet was too limited for such a purpose. The longevity of the flies on sorbitol, however, proved to be so much superior to that on all the other substances tested that we can safely regard this difference as being significant.

Table I. *Nutritional value of sugars and allied substances for the adult blowfly*

Substance		Source	Fed as solid. No. of days after which		Fed in solution. No. of days after which	
			Half are dead	All are dead	Half are dead	All are dead
Pentoses	Arabinose	B.D.H.	.	3½	.	3½
	Xylose	B.D.H.	6	11	5	12
	Rhamnose	Prof. Haworth	.	3	.	3
Hexoses	Glucose	B.D.H.	19	>34	.	.
	Fructose	B.D.H.	20	>34	.	.
	Galactose	B.D.H.	33	>40	.	.
	Mannose	B.D.H.	27	49	.	.
	Sorbose	Prof. Haworth	2	4	.	3
Disaccharides	Sucrose	B.D.H.	30	46	.	.
	Maltose	B.D.H.	18	30	.	.
	Lactose	B.D.H.	.	3½	4	14
	Trehalose	Prof. Helferich	29	44	.	.
	Melibiose	Prof. Haworth	35	.	.	.
	Cellobiose	Prof. Haworth	.	3	.	3
Trisaccharides	Raffinose	B.D.H.	21	.	.	.
	Melezitose	Prof. Zemplen	31	.	.	.
Polysaccharides	Dextrin	B.D.H.	20	.	.	.
	Starch	B.D.H.	.	3½	7	22
	Glycogen	—	3	4	3	15
	Inulin	Dr F. G. Young	.	3	.	.
Glycosides	α-Methyl-glucoside	B.D.H.	26	56	.	.
	β-Methyl-glucoside	Kahlbaum	.	4	.	4
	α-Methyl-galactoside	Prof. Haworth	5	9	15	.
	β-Methyl-galactoside	Prof. Schlubach	.	3	.	.
	α-Methyl-mannoside	Eastman Kodak	.	3	.	3
	β-Methyl-fructo-pyranoside	Prof. Schlubach	.	3	.	.
Sugar alcohols	Mannitol	B.D.H.	16	41	.	.
	Sorbitol	B.D.H.	47	80	.	.
	Dulcitol	B.D.H.	.	2½	.	3
	Erythritol	B.D.H.	.	3½	.	3½
	Inositol	Dr F. G. Young	.	3½	3	4

Group 2. The sugars which had no nutritional value at all were the pentoses arabinose and rhamnose; the hexose sorbose; the disaccharide cellobiose; the polysaccharide inulin; the glycosides β-methyl-glucoside, β-methyl-galactoside, α-methyl-mannoside, β-methyl-fructopyranoside; the phenol-glucosides helicin, alicin, arbutin; and the alcohols dulcitol, erythritol and inositol. In all these cases he flies did not seem to touch the dry substance but appeared to drink freely of an queous solution of it; hence it seems certain that the substance can have no utritional value for these insects.

Group 3. Of the third group of substances, i.e. those on which the length of life vas somewhat increased, xylose was eagerly eaten, but not utilized to a great extent. Lactose was not touched in the dry state, but was taken readily in solution and was tilized to only a limited extent, for the walls of the cages soon became splashed with vhite stains of apparently unaltered lactose, which had passed the gut and had been xcreted as faeces. These particular feeding experiments were repeated twice, sing 100 flies in all so that the results are more reliable than most of the others. It s suggested that the limited degree of utilization of lactose by the fly is due to acterial action in the gut, and not to digestion by enzymes produced in the gut.

The two polysaccharides starch and glycogen were obviously not touched as lry substances, but did maintain life to a limited extent when fed in aqueous olution. The gut of the adult fly contains amylase (Wigglesworth, 1929), but it eems likely from the feeding experiments that this enzyme is present in too weak a oncentration to produce glucose in sufficient quantity for maintaining the life of he fly. It was obvious in the experiments with starch and glycogen that the najority of flies died at an early stage, and only a small percentage were able to live or a considerable period. In one particular experiment, using starch dissolved in vater, the survival of the flies was as follows:

2nd day	9 flies dead (no utilization!)
4th to 8th day	3 flies dead
9th day	1 fly dead
10th day	2 flies dead
11th day	1 fly dead
13th day	1 fly dead
14th day	1 fly dead
22nd day	Last fly dead

Tests were also made to find out to what extent sugars can be replaced by other ubstances. It was assumed that some of these might be converted in the fly rganism to sugars and used as such, which would be manifested by their effect on he survival. In vertebrates, for instance, proteins are known to be convertible to sugars, while there are other substances which are known to be intermediary products f carbohydrate metabolism. The choice of substances for the experiments with lies was thus largely determined by their convertibility into glycogen after injection nto the blood stream of vertebrates.

With regard to the possible utilization of proteins, it has been shown previously (Glaser, 1923; Evans, 1935) that meat, which is the natural food for the adult lowfly, keeps the insects alive for no longer than water alone, and I myself have ound that on a diet of meat and water flies will survive for only 3 days, while on eptone, which probably contains all the essential elements of meat protein, they nay live for 4½ days. Another natural source of protein is milk; on milk alone half he flies were dead after 4½ days, and the last fly died after 17 days. The survival here was no more impressive than on lactose dissolved in water. It can therefore b concluded that proteins are not converted into sugars in the fly. This is not due t the deficiency of the gut of the adult fly in proteinases, as has been claimed b Wigglesworth (1929), for the fact that feeding of proteins is indispensable for th formation of eggs shows that the gut can digest these substances. Furthermore, th presence of the proteinases "trypsin" and "erepsin" can easily be demonstrated with the alkaline casein method (Cole, 1933, Exp. 273) and the biuret test. It would appear, therefore, that the adult fly either cannot produce intermediary carbohydrates from protein, or does so at such a slow rate that it cannot maintain th ordinary muscle metabolism.

Of the substances which are known partly as intermediary products in the carbohydrate metabolism, partly as good glycogen formers in the liver of vertebrates, the following have been tested: glycol, dihydroxyacetone, alanine, glycine butyric acid, *iso*butyl alcohol, lactic acid, pyruvic acid, succinic acid, malic acid citric acid. For the acids the sodium salt as well as the free acids were tested The results of all these feeding experiments were negative. It is possible that this was due to the fact that some of the substances were not eaten by the flies at all, or only in very small quantities; that they acted as poisons; that they were not absorbed in the gut, or that they acted adversely by their high osmotic pressure. No particular precautions had been taken, however, to obviate certain of these effects, so the negative results obtained ought not to be taken as final. But none of these adverse factors seems to be applicable to the experiments with glycine or alanine and the non-utilization of alanine is particularly interesting in view of the fact that this amino-acid, which is closely related to pyruvic acid, is utilized as a glycogen former in mammals (Neubauer, 1928). This observation seems to indicate that the course of carbohydrate metabolism is not identical in insects and vertebrates.

The only non-carbohydrate which was utilized, although only to a slight extent, was glycerol. The following are the protocols of two different feeding experiments, in which an approximately 2% solution of glycerol was supplied:

Number of flies dying on the	Exp. 1	Exp. 2	Control (water)
3rd day	11	—	20
4th day	1	3	—
5th day	5	7	—
6th day	3	3	—
7th day	—	5	—
8th day	—	2	—

There can be no doubt, therefore, that glycerol does prolong the life of the fly and it seems most likely that this is due to the conversion of glycerol into a carbohydrate or one of the intermediary products which would normally arise in carbohydrate metabolism. It is thus possible that after the difficulties of presentation have been overcome some of the other non-carbohydrate substances, which have so far given negative results, may prove to be convertible into and utilizable as carbohydrates by the fly.

DIGESTION EXPERIMENTS

From what we know of the absorption of sugars in the gut of other animals, it is very likely that pentoses, hexoses and sugar alcohols are immediately absorbed from the blowfly gut without any previous change in their structure, but that di-, tri- and polysaccharides and glycosides are only absorbed after they have been broken down to monosaccharides. It is to be expected, therefore, that all the di-, tri- and polysaccharides and glycosides which are utilized by the fly will also undergo hydrolysis in the gut, which must, therefore, presumably, produce all the enzymes which are necessary for this purpose, although the possibility that the breakdown is due to bacterial action must not be overlooked.

To test for the presence or absence of carbohydrases in the gut, digestion experiments were carried out *in vitro*, with di-, tri- and polysaccharides and glycosides as substrates. The enzyme preparations were made from the guts of newly emerged and unfed flies, because in this state the presence of large numbers of bacteria in the gut seemed unlikely. A number of guts were dissected out from the flies, ground up in water with sand and the resulting suspension of gut tissues distributed into small specimen glasses $2 \times \frac{1}{8}$ in., so that each tube contained approximately the substance of one-half of a gut. About 0·3 ml. of a 2% solution of the substrate was added, together with 5 drops of a buffer solution and 1 drop of toluene to prevent bacterial growth. These tubes were incubated for 24–48 hr. at 36° C. and subsequently tested for the presence of glucose, fructose or galactose by the osazone method. Since only 6 mg. of the substrate were needed for each individual test, it was possible, even when the sugars were available only in very small amount, to test over a wide range of *p*H values, the necessary controls being made with gut suspensions in which the enzymes had been destroyed by boiling. An incubation period of 24 hr. proved to be sufficient in all the tests that gave a positive result. The only instance in which the osazone test failed was in the case of β-methyl-fructopyranoside; here glucosazone was formed abundantly not only in the controls with the boiled gut suspensions, but also with the pure substance alone.

The results of these digestion experiments are shown in Table II. The system of registering the intensity of an enzyme action as negative, uncertain, weak, medium or strong by use of the symbols −, ±, +, ++ or +++ is self-explanatory, but the gradation, of course, is purely arbitrary and is based only on a subjective judgement of a result and not on any quantitative determination, which was impossible with the small amount of material available. Comparison of Table II with Table I shows that all the di- and trisaccharides and glycosides which are of great nutritional value for the fly are converted by an enzyme present in the gut into monosaccharides. On the other hand, those substances which have no nutritional value are all unattacked. The only exception to this appeared to be lactose, which proved to be of some slight nutritional value, but for which the presence of an enzyme (lactase) could not be demonstrated. The reason for this may be either that the enzyme is present only in very weak concentration, or that, as has already been suggested, the attack in the gut is due to micro-organisms and not to an enzyme produced by the gut itself.

Table II. *Enzymatic digestion of carbohydrates in the gut of the adult blowfly*

Substance		Chemical composition	Action of enzyme			
			Without buffer	At *p*H 3·6	At *p*H 5·4	At *p*H 7·c
Disaccharides	Sucrose	α-Glucosido-fructose and β-fructosido-glucose	+++	+++	+++	+++
	Maltose	α-Glucosido-glucose	+++	+++	+++	+++
	Lactose	β-Galactosido-glucose	–	–	–	–
	Trehalose	α-Glucosido-glucose	+++	+++	+++	+++
	Melibiose	α-Galactosido-glucose	–	+	+++	+
	Cellobiose	β-Glucosido-glucose	–	–	–	–
Trisaccharides	Raffinose	α-Galactosido-sucrose	–	+	+++	+
	Melecitose	α-Glucosido-sucrose	+++	+++	+++	+++
Glycosides	α-Methyl-glucoside		+++	+++	+++	+++
	β-Methyl-glucoside		–	–	–	–
	α-Methyl-galactoside		–	–	++	–
	β-Methyl-galactoside		–	–	–	–
	β-Methyl-fructo-pyranoside		?	?	?	?
	α-Methyl-mannoside		–	–	–	–

In most cases the enzymatic action of the gut suspensions was very vigorous, as seen by the very abundant formation of glucosazone crystals. Under the conditions of these tests there was no visible difference in the enzyme actions over a wide range of *p*H from 3·6 to 7·0, except in the case of α-methyl-galactoside, which was only hydrolysed at *p*H 5·4. An optimum enzyme action at *p*H 5·4 was also shown in some of the experiments with melibiose and raffinose and since these three substances are α-galactosides, it seems that the range of *p*H for enzyme action is much narrower for α-galactosidases than for α-glucosidases.

DISCUSSION

Simultaneously with the writer's preliminary note on the utilization of sugars by the adult blowfly appeared a paper by Haslinger (1936) dealing with almost the same problem. Haslinger's method of testing differed from mine in so far as each fly was fed by him individually and the various diets contained equivalent amounts of sugars. In order to induce the flies to feed, a constant amount of sucrose was added to each test-solution, which was sufficient to render it sweet for the flies, but insufficient to prolong their life. I have fed all substances in surplus quantity, and induced feeding, where it was necessary, by dissolving them in water. Table III shows that in spite of the difference in method and the small number of flies used in each experiment there is a remarkable agreement between the results of the two independent sets of experiments. The only other work with insects, in which the nutritional value of sugars has been determined by the survival method, was undertaken by Phillips (1927) and Vogel (1931) with adult bees. The results of Vogel's and Haslinger's experiments (Table III), taken from the latter's paper, are expressed in terms of the nutritional value of sucrose, which is taken as unity. The adult bee resembles the adult fly inasmuch as in both insects life can be adequately maintained on sucrose alone, but, with regard to the majority of the other substances, although there is qualitatively a good agreement in their nutritional value for both insects, there are also considerable differences. Mannose not only fails to serve as a diet for the bee, but has, strangely enough, a poisonous effect. Cellobiose and arabinose, which were of no nutritional value for flies, have a considerable value for bees. Xylose has a much greater nutritional value for bees than for flies. On the other hand, galactose, lactose, melibiose and raffinose, all sugars which contain galactose, are of much greater nutritional value for flies than for bees. Melibiose, in fact, has

Table III. *Utilization and digestion of carbohydrates in the adult blowfly, the adult bee,* Helix pomatia *and vertebrates*

		Fly		Bee	*Helix pomatia*	Vertebrates	
		Fraenkel. Utilization and digestion	Haslinger. Utilization	Vogel. Utilization	Bierry. Digestion	Isaac & Siegel. Utilization	Fischer, Bierry. Digestion
Pentoses	Arabinose	–	0	0·8	.	+ ?	.
	Xylose	+	0·1	1	.	+ ?	.
	Rhamnose	–	0	0	.	+ ?	.
	Fucose	.	0	0	.	.	.
Hexoses	Glucose	+ + +	1	1	.	+	.
	Fructose	+ + +	1	1	.	+	.
	Galactose	+ + +	1	0·3	.	+	.
	Mannose	+ + +	0·8	0	.	+	.
	Sorbose	–	.	–	.	+	.
Disaccharides	Saccharose	+ + +	1	1	+	.	+
	Maltose	+ + +	1	1	+	.	+
	Lactose	+	0·1	0	+	.	+
	Trehalose	+ + +	0·8	1	.	.	+
	Melibiose	+ + +	0·4	0	+	.	–
	Cellobiose	–	0	0·5	+	.	–
	Gentiobiose	.	.	.	+	.	–
Trisaccharides	Raffinose	+ + +	0·9	0·12	+	.	–
	Melezitose	+ + +	0·9	1	.	.	.
	Gentianose	.	.	.	+	.	–
Polysaccharides	Dextrin	+ +	.	.	+	.	+
	Starch	+	.	.	+	.	+
	Glycogen	+	.	.	+	.	+
	Inulin	–	.	.	+	.	–
Glycosides	α-Methyl-glucoside	+ + +	0·4	1	+	.	–
	β-Methyl-glucoside	–	.	.	+	.	.
Sugar alcohols	Mannitol	+ + +	0·7	1	.	±	.
	Sorbitol	+ + + + +	0·8	2	.	+	.
	Dulcitol	–	0·1	0	.	±	.
	Erythritol	–	0	0	.	–	.
	Inositol	–	0·1	0	.	–	.

no value at all for bees. It is interesting to note that in two of the feeding trials sorbitol proved to be of considerably higher nutritional value than all the other sugars tested, including sucrose. This phenomenon is so far unexplained, but it may possibly be due to the easier conversion of sorbitol into one of the intermediary products of carbohydrate metabolism after absorption into the blood.

The snail *Helix pomatia* seems to be the only animal in which the presence or absence of carbohydrases in the gut has been tested hitherto for a wide range of substances (Bierry, 1911, 1912; Oppenheimer, 1925, 1935). Here the gut juice has been shown to hydrolyse not only every di- and trisaccharide and glycoside tested, but also polysaccharides of such a resistant nature as cellulose, inulin and chitin.

Almost nothing is known at present about the nutritional value of a wide range of sugars and allied substances for any representative of the vertebrates, nor does a systematic enzyme survey similar to that with *Helix* by Bierry and with the fly by the writer appear to have been undertaken. Such information as is available so far is incomplete and often contradictory, but the data given in the last two columns of Table III, compiled mainly from Isaac & Siegel (1928), Fischer & Niebel (1896) and Oppenheimer (1925, 1935), represent what seems to be the most probable position at the present time. From this it seems that the range of substances which are digested in the vertebrate gut is much smaller than in the invertebrates so far tested, but it should be remembered that most of the present information on the utilization of pentoses, hexoses and sugar alcohols by vertebrates is limited to the part which these substances play in glycogen formation after injection into the blood; it is not known whether the substances are utilized to a similar extent when fed.

A review of feeding experiments with sugar alcohols brings out some strange contrasts. Sorbitol, which on oxidation yields glucose and fructose, is fully utilized by bees and flies, as also is mannitol, which is oxidized to mannose. From this one would expect that dulcitol, which is oxidized to galactose, could be utilized as well, but this has not been borne out by experiment. In the case of vertebrates the position is still more confusing. Sorbitol is fully utilized when injected into the blood, but, strangely enough, not at all when fed (Waters, 1938); mannitol, on the other hand, is not even utilized when injected, nor is dulcitol (Fromherz, 1928).

The present study with blowflies seems to be the only one to date in which feeding experiments have been correlated with *ad hoc* investigations (*in vitro*) of the enzymes concerned; flies are, indeed, extremely favourable material for such a research, since they are small enough for the former purpose yet large enough for the latter. Throughout the investigation the opinion has been expressed that the observed utilization and digestion of those substances which do not pass readily through the gut wall is due to the action of enzymes produced by the gut wall and not by micro-organisms living in the gut lumen. To establish this beyond doubt it would have been necessary to carry through the feeding experiments with sterile flies, and although it is possible to produce aseptic flies by rearing the larvae under aseptic conditions, it seemed impossible to keep them so for periods of weeks while changing the food daily. The main reason for assuming that micro-organisms do not lay an essential part in the effects observed is the close agreement between the esults of the feeding experiments and the enzyme tests *in vitro*, in which sterile onditions were obtained. The action of living bacteria is therefore excluded in the ests.

There remains only the possibility that the gut harbours a large amount of acteria and that the enzyme action observed in the tests was due to enzymes xtracted from them and not from the gut wall. To test this, a count of the bacteria resent in the guts of newly emerged flies was undertaken with the aid of Dr S. E. acobs, to whom I wish to express my sincere thanks. From ten guts a suspension 10 ml.) was prepared by the usual method, during which, admittedly, conditions ould not be kept strictly sterile, and tests were subsequently made by incubation on utritive agar. No bacterial growth took place in those which contained 1 ml. of his suspension. If, however, the latter had been previously diluted tenfold with water 1 ml. produced thirteen bacterium colonies. A hundredfold dilution under imilar conditions gave only two colonies, while more dilute suspensions gave none t all. The gut suspension contained, therefore, 130–200 bacteria per gut. This small umber is almost certainly derived from infestation during the course of the prepara- ion and not from the interior of the gut. The fact that no bacteria developed in the ests with the undiluted suspension suggests that an inhibitor for bacterial growth was present in the gut. It can therefore be said with certainty that the gut suspensions used for the enzyme tests contained so few bacteria that the strong enzymatic action observed *in vitro* must be attributed to enzymes extracted from he gut wall.

Table II shows that of all the di- and trisaccharides and the glycosides tested, t least eight are hydrolysed by enzymes secreted by the gut of the fly. In keeping with older views as to the specificity of enzymes one would have to assume the resence in the gut of eight different and specific enzymes, namely, sucrase, maltase, rehalase, melibiase, raffinase, melezitase, α-glucosidase and α-galactosidase. This ssumption would appear somewhat far-fetched in view of the fact that only two of he sugars concerned, namely, sucrose and maltose (the latter as a breakdown product f starch and glycogen) would occur commonly in the natural food of the flies; and, ndeed, certain of the compounds hydrolysed are not met with at all among natural roducts. On the other hand, two of them that are utilized only slightly, if at all, .e. lactose and cellobiose, are available to flies, the one in milk and the other in decomposing wood. In considering these observations, the recent views of Weidenhagen become of interest. According to Weidenhagen (1931), di- and risaccharides or glycosides are hydrolysed, not by an enzyme specific for each ompound, but by glycosidases whose action is determined by the identity of the ugar whose potentially aldehydic carbon atom enters the glycoside link, and by whether combination occurs through the α- or β-position. This theory postulates he existence of only five enzymes, capable of hydrolysing all known di- and tri- accharides and glycosides based upon glucose, galactose and fructose, namely: -glucosidase, which splits all α-glucosides (sucrose, maltose, turanose, trehalose, melezitose); β-glucosidase, which splits all β-glucosides (gentiobiose, cellobiose, phenylglucosides); α-galactosidase, which splits all α-galactosides (melibiose raffinose); β-galactosidase, which splits all β-galactosides (lactose); and β-h fructosidase, which splits all β-fructosides (sucrose, raffinose, gentianose). Applyin this scheme to our results for the feeding experiments with flies, we find the closes possible agreement. All the α-glucosides (sucrose, maltose, trehalose, melezitos and α-methyl-glucoside) and α-galactosides (melibiose, raffinose, α-methyl-galactoside) tested are digested, but none of the β-glucosides (cellobiose, β-methyl glucoside, phenolglucosides) or β-galactosides (lactose, β-methyl-galactoside Whether β-*h*-fructosidase is present in the fly cannot be decided from thes experiments, since of the fructosides tried sucrose is also split by α-glucosidase an raffinose by α-galactosidase. The fact that β-methyl-fructopyranoside has n nutritional value for the flies is irrelevant, since fructose in sucrose and raffinose i of the furanose type. Whether fructosidase is involved in the breaking up of sucros and raffinose can only be decided by testing with α- and β-methyl-fructofuranoside which I was unable to obtain. All the findings as to the utilization and digestion c di-, and trisaccharides and glycosides may, therefore, be fully explained by assumin the presence of only two enzymes, α-glucosidase and α-galactosidase, in the gut c the fly, and they provide an illuminating commentary on the validity of Weidenhagen's views.

SUMMARY

1. A series of different sugars and sugar alcohols has been fed to adult flies an their nutritional value determined by their effect on longevity.

2. All the di- and trisaccharides and glycosides which are of great nutritiona value for the flies are split in the gut of the flies by enzymes the presence of whic could be demonstrated *in vitro*. No enzymes could be found by the same metho which would split any of the substances which had been shown to have no nutritiv value.

3. Weidenhagen's theory of the specificity of carbohydrases offers a convincin explanation of the results of the feeding and digestion experiments. The presenc of only two enzymes, α-glucosidase and α-galactosidase in the gut of the fly woul account for the different action of various di- and trisaccharides and glycosides.

I am much indebted to Prof. D. M. S. Watson of University College, London in whose Department part of this work was carried out. I have to thank also th Academic Assistance Council for a grant which enabled me to start this investigation and Prof. J. W. Munro who obtained further financial support for it through th Agricultural Research Council. Finally, I should like to thank those whose name are mentioned in Table I for their kindness in providing me with samples of rar substances. Prof. A. C. Chibnall and Dr H. W. Buston were also good enough t read and criticize the manuscript.

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DISK-SPHERE TRANSFORMATION IN MAMMALIAN RED CELLS

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(With Two Text-figures)

THE purpose of this paper is to try to explain the phenomenon of the transformatio of the mammalian erythrocytes from disks to spheres when a small drop of a salin suspension is placed between glass slide and cover-glass. Ponder (1934) severa years ago reviewed the work which had been done in connexion with this phenomenon. As was pointed out by Ponder, who was the first to show clearly th necessity of having the glass surfaces in close proximity for the formation of perfec spheres (1928–9), the two explanations which have been put forth to explain th phenomenon are both inadequate. As will be seen later, that of Waller (1930) i somewhat closer to the true explanation than that of Brinkman & van Dam (1920)

It will be shown that the phenomenon of sphering is a result of two change which occur when the drop of suspension is placed between slide and cover-glass One change is the increase in alkalinity of the suspension because of the alkalin nature of the glass surfaces. The other change is the adsorption on to the glas surfaces of a substance from the cell suspension. If this substance is present in th suspension, it will prevent the formation of spheres at the *p*H attained under th cover-glass, but if it is removed, as by adsorption, the cells will become spheres a that *p*H. Unless otherwise noted, all the cell suspensions used in the experiment about to be reported were suspensions of human erythrocytes in saline, made b adding 0·5 c.c. of whole blood, directly or within a few hours of drawing an oxalation, to 100 c.c. of 1 % NaCl. The observations on the cells of such a suspension were always made within 3 hr. of the formation of the suspension in order t avoid the changes in the red cells which occur on long standing.

Also, unless otherwise stated, the slides and cover-glasses used in the experiments were of new glassware, wiped clean with a soft towel prior to being used Several brands and grades of slides and cover-glasses were tested, but all responde the same as far as the sphering was concerned.

ALKALINE EFFECT OF SLIDES AND COVER-GLASSES

The possibility of an increase in *p*H of the suspension between slide and cover-glass was put forward by Waller (1930), who, however, presented no positive evidence for such a change. This possibility was first suggested to me when I found that the addition of powdered soft glass to a saline suspension of red cells in such a quantity as to make the ratio of glass surface to volume of suspension comparable to that obtained with a drop of suspension between slide and cover-glass, increased the *p*H of the suspension from approximately 7·0 to over 9·5; the red cells in a suspension so treated became spherical.

In another experiment a microscopic crystal of salicylic acid was placed beneath the cover-glass with a drop of cell suspension. On observing the cells beneath the cover-glass it was seen that all were spheres as usual except those in a ring around the crystal. In this ring, which gradually expanded (apparently as the acid diffused out from the crystal), the cells changed from spheres to crenated forms to disks before undergoing lysis by the acid. This experiment also suggested that an alkalinity increase (which was counteracted by the salicylic acid) between slide and cover-glass played an important part in the sphering phenomenon.

Indicators were used in the first attempts to measure the *p*H in suspensions between slide and cover-glass. Using cresol red, it was found that saline suspensions between slide and cover-glass had their *p*H shifted completely through the colour-changing range of the indicator (7·2–8·8). Moreover, the shift in *p*H appeared to occur almost immediately. This was well in accord with the observation that when a drop of a cell suspension is touched to the edge of a dry cover-glass resting on a slide and is drawn rapidly into the space under the cover-glass by capillary action, the cells become spheres almost as soon as the suspension enters the capillary space.

If the alkaline effect of slide and cover-glass plays such an important role in the sphering of red cells, one would, of course, not expect sphering between slide and cover-glass having no alkaline effect. Such slides were first obtained by cleaning the new slides in chromic acid cleaning solution. However, slides and cover-glasses so cleaned showed with indicators a slight acidifying effect on the saline suspension, even though they were rinsed well after cleaning. True enough, however, the cells in a drop of suspension between such acid-cleaned glass surfaces were not spheres, but were disks and cups.

Success in obtaining neutral reacting slides and cover-glasses, as far as the indicator method was able to detect, was finally achieved by boiling the new slides and cover-glasses for about 15 min. in distilled water and then rinsing them thoroughly in distilled water. Between a slide and cover-glass tested in this manner the cells in a drop of suspension, which had been disks in the bulk of the suspension, did not become spheres, but retained their disk shape. On standing awhile the cells under the cover-glass would often crenate a little or become cup-shaped, or in general, change somewhat from the good biconcave disk shape which they exhibited in the bulk of the saline suspension, but they never became spherical.

Exactly similar results were obtained with a quartz slide and quartz cover-glass as were obtained with the boiled slides and cover-glasses.

By rinsing new slides and cover-glasses for a few minutes with water before using, glass surfaces were obtained which had an intermediate alkaline effect. Between such slides and cover-glasses the cells were neither good disks nor good spheres, but were crenated discoids and spheroids. Such forms, as Ponder (1934) has shown, are the intermediate forms between the biconcave disks and the spheres formed under cover-glass. Such forms were observed by Ponder in intermediate depths of a saline cell suspension placed under a long cover-glass resting on a slide at one end and raised above the slide by a small glass strip at the other end (thus forming a wedge-shaped chamber with the slide). At the narrow end of the chamber Ponder observed spheres, at the deep end he observed discoidal forms, and between the two he observed the gradual gradation of crenated forms. By using the indicator cresol red in such a wedge-shaped chamber, it was seen that the *p*H within the chamber gradually changed from about neutral at the deep end of the chamber to a *p*H of 8·8 or higher at the narrow end of the chamber.

On using the transparent plastic methyl methocrylate as a slide and cellophane as a cover-glass, such a decided acidic reaction was given to the drop of cell suspension that much haemolysis occurred. Prior to haemolysis under these conditions, the cells became first cups and then large prolytic spheres, such as are found when one haemolyses cells with acid in the bulk of a suspension.

Rinsing the new slides and cover-glasses in organic solvents, such as ether, alcohol, petroleum ether, and benzene, had no effect on the ability of them to cause the formation of spheres.

To study the phenomenon of the disk-sphere transformation further it was desirable to determine the *p*H in drops of suspension placed between slide and cover-glass much more accurately than could be determined with indicators. A method for doing this with the use of a micro-glass electrode was therefore developed. A description of the apparatus and technique follows.[1]

The glass electrode was made by a modification of the method of MacInnes & Dole (1929). The tube to which the membrane was to be attached was drawn out in the form of a medicine dropper. The smooth tip, with an internal diameter of about 1·0 mm., was plunged while at a dull red heat into an irridescent bubble of Corning 015 electrode glass. If the fusing of the membrane to the tip was successful, 0·1 *N* HCl was poured into the sealed tube. Into this acid a silver-silver chloride electrode, prepared by the method of Brown (1934), was placed. The membrane was submerged in water for about a day before being used and was always kept submerged in water when not in use. This micro-electrode was used in conjunction with a regular macro-glass electrode potentiometer (model 3 C of the Coleman Electric Co.). To replace the cup as a receptacle for the suspension with *p*H to be measured, a flat-topped glass tube with a small capillary opening from the large bore of the tube through the centre of the flat top was used (see Fig. 1). The KCl bridge leading from the regular calomel half-cell of the apparatus passed up through the tube to the top of the capillary. On top of the tube and therefore in contact with the KCl solution in the capillary was placed a drop of the suspension of which the *p*H was to be measured. The tip of the micro-glass electrode was placed in the drop and the potentiometer balanced out and the *p*H obtained in the usual way. Both micro-electrode and the tube for supporting the drop were conveniently held in clamps on an iron stand. Before each new *p*H determination the glass electrode was rinsed off with distilled water, the capillary was flushed out with KCl solution, and the top of the tube was rinsed off with water and then dried by lightly touching with a filter paper. This apparatus was capable of determining the *p*H of a drop of suspension of as small a volume as 0·005 c.c. with an error of less than 0·05 of a *p*H unit.

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[1] I wish to acknowledge here the suggestions of Dr L. S. Fosdick of the Department of Chemistry, School of Dentistry, North-western University, on the making of the apparatus.

In determining the effect of slide and cover-glass on the *p*H of a drop of suspension with this apparatus, a drop of measured volume was first put on a slide from a micropipette, and a cover-glass of 24 by 60 mm. was placed upon the drop and allowed to remain in place for a half-minute or so. Then the cover-glass was removed by lifting from one end in such a manner that most of the suspension under it was collected in one drop on the slide. This drop was then transferred from the slide to the top of the tube containing the KCl bridge by inverting the slide and touching the drop to the top of the tube. The *p*H of the drop was then determined.

The results of the *p*H determinations with the micro-glass electrode are contained in Table I. It will be noted that observations were made not only of the effect of new slides and cover-glasses on the *p*H of saline suspensions, but also of the effect of boiled slides on saline suspensions, and of the effects of new and boiled slides on buffered suspensions. The *p*H values listed are all averages of several determinations on different drops of suspension.

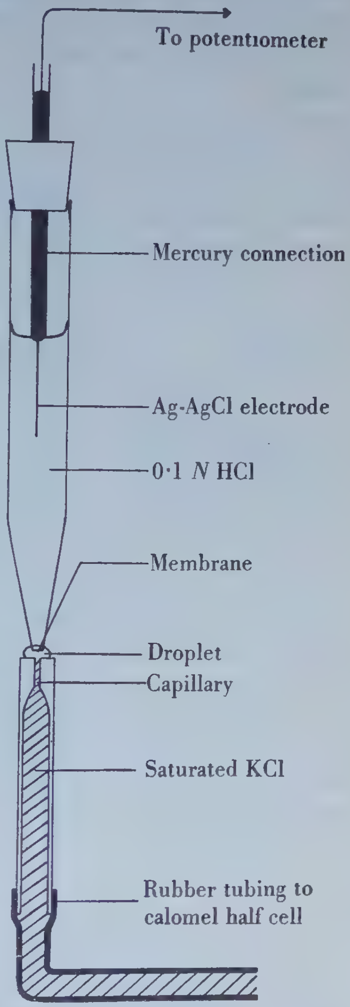

Fig. 1. Micro-glass electrode.

The alkaline effect of the new slides and cover-glasses is surprisingly large. Even the values around 9·2 and 9·3 listed in the table as occurring in drops from between new slides and cover-glasses, which have caused complete sphering, are probably a few tenths of a *p*H unit below those actually attained between slide and cover-glass, for in the process of transferring the drop to the flat top of the tube there was always probably a decrease in *p*H due to the absorption of CO_2 from the air by the drop. This was indicated by the fact that the shorter was the interval of time between removing the new cover-glass from the saline suspension and the measuring of *p*H, the higher was the *p*H generally.

From the table it will be noted that the effect of the boiled slides and cover-glasses on a saline suspension does not appear to be a perfectly neutral one. The lowering of pH in the case of boiled slides and cover-glasses, however, was so littl that it might well have been due to absorption of CO_2 from the atmosphere by th drop in the various steps. Also it will be noted that even in the presence of considerable phosphate buffer the new slides and cover-glasses exerted a marke alkaline action. With a suspension containing 19 parts of saline to 1 part of $M/1$ phosphate buffer, the smooth spheres under the new cover-glass generally occurre in the thinnest depths of liquid, while the crenated spheres occurred in the thickes depths.

Table I

Nature of suspending solution	Nature of slides and cover-glasses	Volume under cover-glass in c.c.	pH of bulk of suspension	pH of drop from under cover-glass	Shape of cells under cover-glass
1% NaCl	New	0·03	7·21	9·20	Perfect spheres
1% NaCl	New	0·02	6·74	9·31	Perfect spheres
1% NaCl	Boiled	0·03	6·70	6·48	Disks, some crenated and cupped
19 : 1 saline to $M/15$ phosphate buffer	New	0·01	7·08	8·50	Spheres and finely crenated spheres
4 : 1 saline to $M/15$ phosphate buffer	New	0·01	7·08	7·87	Finely crenated spheres
9 : 1 saline to $M/15$ phosphate buffer	Boiled	0·02	7·08	6·94	Crenated disks

Two short experiments conclude this section on the alkaline effect of slides an cover-glasses. In the first it was found that by spreading a small drop of a saline suspension of red cells very thinly over a new glass slide the cells changed to spheres even though no cover-glass was placed over them. In the second the cells in a drop of suspension were all changed to spheres by placing the drop between cover-glass and slide. The cover-glass was then removed so as to leave the drop containing the spheres on the slide. After this a comparatively small drop of untreated suspension containing cells in discoidal form, was added to the treated drop on the slide. On the addition the newly added cells also turned to spheres.

ADSORPTION OF ANTI-SPHERING FACTOR

Up to this point has been presented the experimental evidence for the decided alkaline effect of new slides and cover-glasses on drops of suspension between them and the important relation of this effect to the phenomenon of the sphering of erythrocytes in the drops. However, it would be incorrect to assume that the sphere formation is due to the rise in pH alone unless one could obtain spheres by merely increasing the pH of a saline suspension of red cells in bulk to the pH attained by a drop under a new cover-glass. And as it turns out this is not the case.

In the following experiments, which show that a second change accompanying the pH change is necessary for the sphering phenomenon to occur, a number of titrations had to be performed on cell suspensions. The standard procedure for a titration was to put 5 c.c. of the cell suspension in the cup of the glass electrode apparatus (the macro rather than micro) and to add 0·15 *M* NaOH directly to this from a small burette. After each addition of alkali the *p*H of the suspension was determined with glass electrode, and about 0·10 c.c. of the suspension was transferred in a pipette from the suspension to the concavity of a hanging-drop microscope slide, where the shapes of the cells in the transferred suspension were observed with a microscope. A titration was always performed as rapidly as was conveniently possible.

When such a titration was performed on a saline suspension made in the usual way (1 : 200 of blood to saline), complete sphering did not occur until a *p*H of 11·3–11·4 was reached. This was definitely a much higher *p*H than could be obtained through the action of slide and cover-glass. Also the spheres which were finally formed at the high *p*H were unlike those formed under cover-glass in that they were larger in volume and were definitely prolytic spheres, always occurring just prior to haemolysis by the alkali.

The result of the titrations on saline suspensions of whole blood suggested that in such suspensions there was some substance which prevented the cells in the bulk of suspension from becoming spheres at the *p*H levels occurring between new slides and cover-glasses, that such a substance was removed from a drop of suspension between slide and cover-glass by adsorption on the glass surfaces, and that if the substance could be removed from red cells in the bulk of a suspension, the cells would become spheres at the *p*H levels occurring between new slides and cover-glasses. For the sake of convenience the hypothetical substance was called the "anti-sphering factor". If it actually were adsorbed on the glass surfaces of slide and cover-glass in the sphering phenomenon, such action would be compatible with the properties of serum proteins. The possibility of such a protein anti-sphering factor was further suggested by Ponder's surmise (1934) that it is an albumin fraction of the serum which causes the retransformation from spheres to disks when one adds a little serum to a drop of suspension in which the cells have previously been changed to spheres by placing the drop between slide and cover-glass.

At first, on the assumption that the anti-sphering factor was contained in the serum alone, attempts were made to free the cells of the factor by washing them free of serum. The washings with saline were performed with the use of a centrifuge. After the washings, the cells were resuspended in saline in a concentration equal to that of cells in a suspension made by adding 1 part of whole blood to 200 parts saline. However, on titrating suspensions of washed cells with 0·15 NaOH, it was found that the cells still would not become perfect spheres in bulk until a *p*H of about 11·3 was reached. It was noticed that the anti-sphering activity (the determination of which will be discussed later) of the suspension of washed cells was not so great as that of a suspension of whole blood, but there was still a decided amount of anti-sphering activity even in a suspension of cells which had been washed up to ten times with a hundred times their volume of saline each time.

Evidently the cells, for practical purposes, could not be washed free of the anti-sphering factor. Perhaps, however, the changes occurring between slide and cover-glass could be more exactly matched in the bulk of suspension; perhaps the cells in suspension could first have the factor adsorbed away from them by introducing into a suspension of washed cells a comparatively large amount of glass surface prior to the increase of alkalinity. It had already been noted that the cells of a suspension which were not sphered when the pH of the bulk of suspension was raised to about 10, became perfect spheres when a drop of the suspension at that pH was placed between boiled (and therefore practically neutral reacting) slide and cover-glass.

To verify the hypothesis that an anti-sphering factor, incompletely washable from the cells in bulk, might be completely adsorbed from the cells in bulk, the following technique was developed after a number of initial experiments. A dilute saline suspension containing the thoroughly washed cells from about 0·10 c.c. of blood was added to a 500 c.c. beaker full of glass beads. The volume of suspension was enough just to cover the beads. The beads were about 3 mm. in diameter, and prior to use had been cleaned by overnight standing in saturated Na_3PO_4 solution, thoroughly rinsed with water, and dried. As could be shown, they caused no appreciable change in the pH of the suspension of cells added to them. After adding the suspension, the beaker was kept in the ice-box 2–3 hr. The suspension was then decanted off through a cheese cloth. The cells in a suspension of this sort were generally crenated disks when the pH was around neutrality. However, on titrating such a suspension in the usual way, the cells all became perfect spheres at a pH between 9·15 and 9·25. Here, then, was a suspension in which the cells all became spheres at a pH level which actually was reached in drops of suspension between slide and cover-glass. The spheres, too, were in all respects similar to those obtained under cover-glass, and were unlike the larger prolytic spheres obtained around pH 11·3 in the bulk of untreated suspensions. On raising the pH of a bead-treated suspension above 9·2, the spheres persisted until a pH above 11 was reached, when they became larger prolytic spheres and haemolysed.

Now, if to a suspension of red cells which had been treated with glass beads a small amount of serum was added, the cells no longer became spheres at a pH of about 9·2, but behaved exactly like a suspension of untreated cells, not becoming spheres until a haemolysing pH of about 11·3 was attained. Also, if a diluted saline solution of serum, which when ordinarily added to a suspension of treated cells prevented them from being sphered below a pH of about 11·3, was first treated with a large quantity of powdered quartz so that all of the protein in it was adsorbed, it no longer prevented the cells from being sphered at a pH of 9·2 on being added to a bead-treated suspension.

Experiments of the types just described definitely indicate an anti-sphering factor which has to be removed from the red cells of a saline suspension before they will become spheres at a pH around 9·2. Treatment of a cell suspension with large quantities of glass beads evidently results in the anti-sphering factor being removed from the cells by adsorption on the very large surface of the beads.

All the titration experiments so far described were done on suspensions of human red cells in 1 % NaCl solution. For comparative purposes some experiments were also performed on human cells in saline and phosphate buffer mixtures, and on the cells of a few other mammals in saline. It was found that unwashed human cells in mixtures of 9 : 1 and 4 : 1 saline to *M*/15 phosphate buffer (*p*H 7·2), became complete spheres at a *p*H of about 11·1–11·2. Human cells deprived of anti-sphering factor, however, in such saline-buffer mixtures became spheres at a *p*H level several tenths of a *p*H unit below the sphering level of about *p*H 9·2 found with pure saline suspensions.

Beef, dog, and horse cells all behave very similarly to human cells. In the presence of the anti-sphering factor in saline suspension they could not be changed to perfect spheres until just before haemolysis in the *p*H range of 11·2–11·4. Likewise, in the absence of the anti-sphering factor in saline suspension, these cells of different species all first became perfect spheres at a *p*H of about 9·2. Moreover, it was found that any one of the four mammalian sera investigated would serve as a source of active anti-sphering factor for a suspension of any of the other species of mammalian cells which had been previously deprived of their own anti-sphering factor.

INTERMEDIATE SHAPES OF RED CELLS

As has been shown by Ponder (1934), red cells undergoing the change from disk to sphere under a cover-glass pass through certain intermediate crenated shapes. The shapes which they successively pass through in the transformation may be arbitrarily listed as smooth disk, crenated disk, crenated discoid, crenated spheroid, finely crenated sphere, and smooth sphere. All of these intermediate forms could be observed when I gradually raised the *p*H, say by a few tenths of a *p*H unit per minute, of a cell suspension free of anti-sphering factor from a *p*H of about 6·0–7·0 to a *p*H of over 9·2. Indeed, with cell suspensions free of anti-sphering factor, crenated disks most often appeared even in neutral solutions. Crenated discoids appeared about *p*H 8·0, crenated spheroids about *p*H 8·5, finely crenated spheres about 9·0, and perfectly smooth spheres about 9·2. The smooth spheres swelled into prolytic spheres around *p*H 11·2. A diagram of these successive changes is given in row *A* of Fig. 2.

In row *B* of Fig. 2 is shown the shapes successively obtained when I gradually increased the *p*H of a suspension of whole blood in saline, which had not been deprived of any of its anti-sphering factor by washing or adsorption. It will be noted that smooth biconcave disks did not persist from neutral *p*H to the *p*H of prolytic sphering above 11, but that over a wide range of *p*H from slightly above neutral to almost 10, the cells were crenated disks and discoids. Then as one raised the *p*H into the region over 10, the cells surprisingly became smooth disks again. Around *p*H 11 the smooth disks generally tended to cup somewhat and in other ways became distorted. Before becoming swollen prolytic spheres in the vicinity of 11·2 they often passed through cup shapes.

If the cells were titrated with pure serum as a suspending medium sphering and lysis again set in about 11·4, but the cells remained smooth biconcave disks at all the *p*H levels from neutrality to over *p*H 11·0.

Rows *C*, *D* and *E* of Fig. 2 diagram what happened when cell suspensions containing varying amounts of anti-sphering factor were subjected to a sudden rise in *p*H from the region around neutrality to the region between 10·2 and 10·7 by a single addition of the proper amount of 0·15 *M* NaOH. The sudden increase enabled one to see a marked tendency of the cells to form spheres even in the presence of anti-sphering factor, which could not be clearly observed when one gradually raised the *p*H as already described. Row *C* represents the changes which occurred when a suspension of whole blood in saline was titrated. Immediately after the addition of NaOH and rise in *p*H, the cells became crenated spheres. But then, within a few minutes of the addition, the crenated spheres changed to the type of smooth disk which would have been obtained at the same *p*H had the *p*H increase been a gradual one.

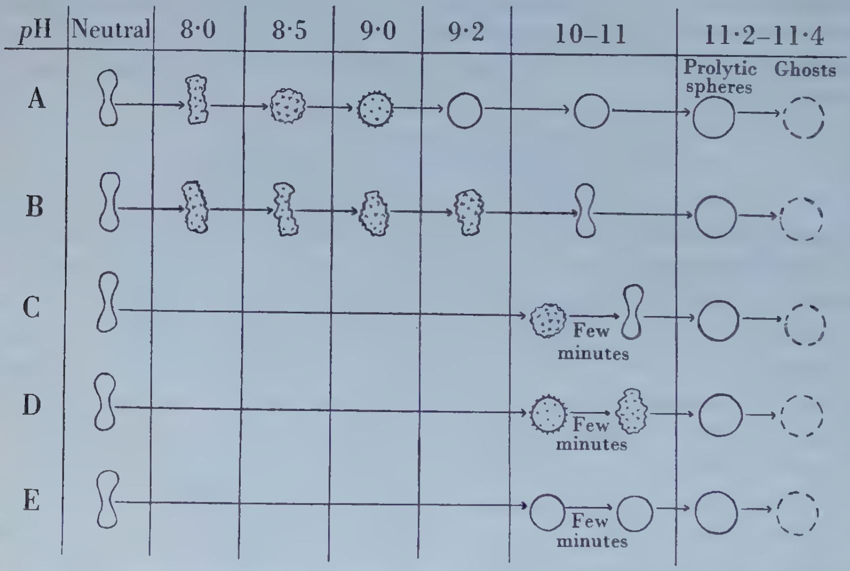

Fig. 2. Shape changes of red cells. *A*, gradual increase in *p*H in suspension free of anti-sphering factor. *B*, gradual increase of *p*H in suspension containing sufficient anti-sphering factor. *C*, sudden increase in *p*H from neutrality to about 10·5 in suspension containing sufficient anti-sphering factor. *D*, sudden increase in *p*H from neutrality to about 10·5 in suspension containing intermediate amount of anti-sphering factor. *E*, sudden increase in *p*H from neutrality to about 10·5 in suspension completely free of anti-sphering factor.

Row *E* represents changes which occurred when a suspension of washed cells which had been completely freed of anti-sphering factor by the glass bead treatment was subjected to a sudden increase in *p*H. Here, however, even after several minutes of waiting, there was no reconversion from the smooth spherical shape which had been formed immediately upon the addition of the NaOH. Row *D* represents the changes occurring when a washed suspension of cells which had been treated with considerably less beads than the suspension of row *E* was subjected to a similar sudden increase of *p*H. Apparently the bead treatment had still left some anti-sphering factor with the cells in this case. In the first sudden change the cells became good spheres except for very fine crenations, but on standing a few minutes they changed to crenated discoids.

I used this rapid titration method in roughly estimating the amount of anti-sphering activity of a cell suspension. The shapes of the cells was observed about 5 min. after the sudden *p*H rise. The less was the amount of anti-sphering factor, the closer to the spherical shape and the farther away from the discoidal shape were the cells. It must be pointed out, however, that any intermediate shape described here as occurring in a suspension under given conditions was not the shape of every cell in the suspension, but rather the shape of the majority of cells in the suspension. Some individual cells often appeared to retain a disk form more persistently than others, while other cells often tended to form spheres more readily than the rest. It was also of prime importance that the cell suspensions be as fresh as possible for one to obtain consistent results.

INFLUENCE OF FATTY ACIDS

At one period in the course of this research reported here I was much troubled by some apparently very inconsistent results. Some suspensions of cells, which had been washed several times, retained considerable anti-sphering activity, while other suspensions, washed just as many times, showed very little anti-sphering activity, and sometimes no anti-sphering activity at all. After some time the cause for the inconsistent results was determined. It happened that when mixing fresh saline with red cells in the washing process, I often used the ball of my palm as a stopper for the centrifuge tube while shaking. In the washing of other batches of cells, however, the mixing was accomplished either by stirring with a glass rod or by rotating the cup rapidly with the hands. Those cells which showed very little or no anti-sphering activity after washing were those which had been mixed with saline in tubes stoppered by the palm. Those cells which showed considerable anti-sphering activity after washing were those which had been mixed otherwise. It therefore seemed that in the mixing against the palm some substance (or substances) passed from palm to cell suspension and counteracted the action of the anti-sphering factor.

To test this idea three control experiments were performed. In one it was found that a 5 c.c. suspension of washed cells, showing considerable anti-sphering activity to begin with, could be completely deprived of anti-sphering activity by shaking it for a few minutes in a 50 c.c. centrifuge tube stoppered with the palm of the hand. In the second it was found that another similar 5 c.c. portion was not noticeably deprived of any anti-sphering activity by shaking it for several minutes in a 50 c.c. centrifuge tube stoppered with a cork. In the third it was found that another similar 5 c.c. portion of unshaken saline suspension, when mixed with a 5 c.c. portion of cell-free saline which had been previously shaken several minutes against the palm of the hand, lost all its anti-sphering activity. Mixing with saline shaken in a tube with a cork stopper, however, caused no noticeable reduction of anti-sphering activity.

A suspension of cells deprived of anti-sphering activity by the shaking agains the palm behaved similarly to a suspension deprived of the anti-sphering factor b treatment with glass beads. The cells first became spheres at a *p*H of about 9· They haemolysed at a *p*H above 11·0. On the addition of a little extra serum to th suspension, the suspension would again show anti-sphering activity, evidently as result of the anti-sphering factor in the serum being added in greater quantity tha could be counteracted by the substance from the skin.

On the hypothesis that the "anti-anti-sphering factor" coming from the ski of the hand was one of the nature of fatty acids, the effects of addition of trace of oleic and palmitic acids to washed and unwashed cell suspensions, originall containing considerable anti-sphering activity, was investigated. The suspension treated with either of these acids also were deprived of their anti-sphering activit and behaved in all respects like suspensions deprived of anti-sphering activity b shaking against the palm.

BEHAVIOUR OF GHOSTS

Concentrated suspensions of washed red cells free of the anti-sphering facto were mixed with distilled water. After the cells had completely laked, the suspen sions were made isotonic again by the addition of the proper amount of 7 % saline The ghosts were washed a few times in saline with a high-speed centrifugation an then were suspended in saline. The amount of haemoglobin remaining in thes watery ghosts depended on the concentration of red cells in the hyptonic haemo lysing mixture, but whether very little or comparatively much haemoglobi remained, the ghosts all behaved in the same manner.

The ghosts in neutral saline suspension were generally disks or crenated disks On placing a drop of a suspension between new slide and cover-glass, the ghosts al became spheres just like unlysed red cells. But within a minute of the initial spherin all the ghosts had changed back to discoidal forms. If unlysed red cells wer placed under the cover-glass with the ghosts, they became spheres with the ghosts but persisted as spheres after the ghosts had changed back to discoidal forms Between boiled slides and cover-glasses the ghosts showed no temporary sphering

The changes of shape of watery ghosts brought about in a drop of suspensio between new slide and cover-glass could also be brought about in the bulk o suspension. By adding enough alkali to raise the *p*H of the suspension suddenl from about neutrality to about 9·5, one could obtain the exact temporary formatio of spheres seen under the cover-glass. This temporary formation of spheres some what resembled the temporary formation of crenated spheres caused by rapidl increasing the *p*H in a suspension containing unlysed cells in the presence of anti sphering factor (see Fig. 2). However, in the case of the unlysed cells the reconver sion to disks was brought about by the presence of the anti-sphering factor, wherea the factor was absent from the suspensions of ghosts. After the ghosts had change back to disks, they could not be changed to spheres again by lowering the *p*H bac toward neutrality nor by raising the *p*H up to about 10·5. Over *p*H 10·5 many o the ghosts appeared to disintegrate and disappear from view. The few fragment which could still be detected at higher *p*H levels appeared to form spheres around *p*H 11·3. If the *p*H of a suspension of ghosts was gradually raised from neutrality to above 10·0, there was no temporary sphering of all cells at once, but in the region around *p*H 9·0, there was some tendency for the crenated forms to become somewhat spheroidal for a short while.

REVERSIBILITY OF DISK-SPHERE TRANSFORMATION

Upon alternately raising and lowering the *p*H of a red cell suspension free of anti-sphering activity from a neutral *p*H to over *p*H 9·2, and from over *p*H 9·2 back to neutrality, the disk-sphere transformation was found to be reversible. When the *p*H went over 9·2 the cells became spheres; when it was reduced to neutrality they again became disks. In changing again to disks they passed through, in reverse order, the same intermediate crenated forms they had passed through on the way to spheres. By shifting the *p*H back and forth with the use of 0·15 *N* NaOH as the base and either 0·15 *M* HCl or CO_2 gas as the acid, the cells could be made to go back and forth through the transformation an indefinite number of times. With long continuation of this increasing and decreasing of *p*H, the *p*H level of complete sphering generally fell several tenths of a *p*H unit below 9·2.

The reversibility of the disk-sphere transformation could also be demonstrated on a new glass slide, using the alkaline glass surface as the source of base. A drop of suspension was put between new slide and cover-glass in the usual way, so that spheres were formed. The cover-glass was then removed so as to leave the drop of saline containing the spherical cells on the slide. Then if a small jet of CO_2 was directed over the drop, all the cells suddenly changed to disks. On withdrawal of the source of CO_2, the cells all changed back to spheres. These spheres could again be changed to disks by blowing on more CO_2. The number of reversals of shape one could produce this way in a single drop was only limited by the evaporation of a drop. In some preparations the transformation from sphere to disk could be brought about by merely blowing the breath strongly on the drop. Agitation of a drop with a metal wire or with a blast of pure air would not bring on such a transformation. Evidently, the addition of CO_2 at first lowered the *p*H of the drop enough to cause the transformation to disks; but on short standing after CO_2 treatment, a *p*H above the sphering level *p*H was again attained because of the reduced CO_2 tension and the counteracting of the acid effect of the added CO_2 by more alkali from the glass and possibly to some extent by the haemoglobin in the cells.

By using the technique just described, an observation was made which has some bearing on the question of whether the red cell has any fixed structure. If I carefully observed the cells in a series of disk-sphere reversals, I could see that *any one cell always had its biconcavities reappear in the same parts of its surface in changing from perfect sphere to disk* (easily noted in the case of cells stuck on edge to the glass slide), and that even the crenations in the transitional forms always reappeared at the same points on the cell surface. Individual cells, however, appeared to have individual differences as far as the exact shape of the crenated transitional forms went.

OTHER PHENOMENA

As already mentioned, the observations of the shapes of cells in a suspension being titrated in bulk were made on about 0·1 c.c. portions of the suspension transferred by pipette to the concavity of a hanging-drop microscope slide. The shapes reported in the previous sections were the shapes occurring toward the middle of the drop under observation in the concavity. In the case of red cells free of anti-sphering factor and in the case of ghosts, the shapes toward the middle of a drop were about the same as the shapes of cells near the surface. But in the case of suspensions of washed and unwashed cells containing anti-sphering factor the shape of the cells within a few micra of the surface of the drop were different from the shape of those in the bulk of the drop. As the *p*H was raised gradually, those cells near the surface successively took on the shapes which those cells in a suspension free or almost free of anti-sphering factor would take on under a similar *p*H change. That is, they tended to sphere at *p*H levels above 9·2, while the cells below them in the bulk of the drop were in discoidal shapes. I suggest that this tendency toward sphere formation at the surface of a suspension containing anti-sphering factor is a result of the adsorption of the anti-sphering factor away from the cells near the surface on to the suspension-air interface.

Occasionally some of the cells in a suspension, after treatment with glass beads, would become crenated discoids, which, despite the removal of the anti-sphering factor, would remain crenated discoids even when the *p*H was raised above the sphering level. In fact, these cells would not even show a temporary tendency to sphere with a sudden rise in *p*H. Evidently, they had somehow been altered in the treatment for removing the anti-sphering factor.

In contrast with these cells which never showed a tendency to sphere were the spherical cells formed in blood which had stood a long time after drawing. Such cells never showed a tendency to acquire any other shape than the spherical, no matter how much anti-sphering factor was present or at what level in the range between acid and alkali haemolysis was the *p*H.

DISCUSSION

The investigation of the causes of the transformation of red cells from disks to spheres in a drop of saline between new slide and cover-glass has led to the realization that there are two important factors influencing the shape of red cells whether between slide and cover-glass or in the bulk of a suspension; namely, the hydrogen-ion concentration of the suspension and the amount present of a substance which militates against sphering. Taking these two factors into consideration, one is able to explain certain hitherto inexplicable findings of former investigators relative to the sphering phenomenon.

The reason why one does not obtain complete sphering if the depth of suspension between slide and cover-glass is too great (Ponder, 1928–9) is that the depth depends on the ratio of the volume of suspension to area of glass surface in contact with the suspension; this ratio must be small in order for the glass surface to adsorb all the anti-sphering factor from the suspension and to furnish enough alkali to raise the *p*H of the suspension above the sphering level.

Cells will not become spherical between slide and cover-glass coated with paraffin (Ponder, 1928–9) because such slides and cover-glasses can neither adsorb anti-sphering factor nor give off alkali. If enough serum is in the suspension under the cover-glass the cells will not become spheres because there is more anti-sphering substance present than can be adsorbed and probably also because the serum exerts a buffering action against the alkaline effect. The ability of ammonium oxalate (Gough, 1924) and phosphate buffers of *p*H less than 7·0 (Waller, 1930) to act somewhat like serum in changing cells sphered beneath a cover-glass back to discoidal shapes is due to their ability to buffer the alkali from the glass. The lack of sphering between slide and cover-glass coated with calcium stearate monolayers (Ponder & Neurath, 1938) may be due to a loss of alkalinity with the formation of the new surface and the inability of such a surface to adsorb the anti-sphering factor.

I regard as completely untenable the hypothesis of Brinkman & van Dam (1920), which attributes the sphering phenomenon to the electrostatic charge on the glass surface and the lack of cholesterol in the cell suspension. Like Ponder, I have been unable to confirm the reports that flaming the slides (Kesten & Zucker, 1928–9; McGlone, 1926) or washing them with ether and drying them in air (Waller, 1930) affected their ability to cause sphere formation. Perhaps as Ponder & Neurath suggest (1938), the ether used by Waller was impure and left a hydrophobic layer on the glassware. Such a layer might prevent adsorption of the anti-sphering factor. There is also the possibility that the ether used by Waller contained certain acidic impurities which neutralized the alkali of the glass surface. This possibility is suggested in view of the fact that one sample of ethyl alcohol which I used for rinsing some of my slides and cover-glasses definitely did away with the alkaline effect of the glass surfaces.

From the results of work now in progress it appears that the anti-sphering factor is very likely the carbohydrate free fraction of the serum albumin.[1] It seems that this protein is in equilibrium between red cells and serum, and when the serum is washed away, it is in equilibrium between cells and saline. However, the red cells hold so tenaciously to what anti-sphering protein is left them after the removal of the serum, that in the equilibrium between cells and saline very little of the protein is in solution in the saline. This accounts for the inadequacy of repeated washings to remove all of the anti-sphering protein from cells. When, however, one brings a saline suspension of cells in contact with a comparatively large area of clean glass surface, as when placing a drop between slide and cover-slip, the anti-sphering protein molecules in solution coming in contact with the surface are irreversibly adsorbed. With the protein being removed from solution by adsorption more protein leaves the red cells to enter the solution in the attempt to establish an equilibrium. This also is adsorbed in turn, and very soon all the protein is thus adsorbed away from the cells on to the glass surface.

As to the actual physical and chemical changes in the red cell which result in changes in shape when one alters the hydrogen ion or anti-sphering factor content of a suspension, nothing can be said at present, but attention should be called to the observation that in repeated reversing of the disk-sphere transformation the biconcavities and particular crenations of any one cell always reappear on the same parts of the surface of that cell. This is strong evidence for some sort of fixed cellular structure.

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[1] The work being done on the determination of the chemical nature of the anti-sphering factor will be reported in a future paper.

SUMMARY

1. Experimental evidence has been presented to show that the sphering of mammalian red cells in saline suspension between new slide and cover-glass is a result of these two changes: (1) the *p*H of the suspension is raised over 9·2 by the alkaline effect of the glass; (2) the glass adsorbs out from the suspension a protein which, if present, prevents sphering until a *p*H of about 11·3 is reached, but if absent allows sphering around a *p*H of 9·2.

2. Anti-sphering factor is contained by both serum and cells. It cannot be adequately removed from cells by washing them with saline, but it can be removed from them by adsorption on clean glass surfaces.

3. Changes of cell shape occurring between slide and cover-glass may be duplicated in the bulk of a suspension.

4. The *p*H and amount of anti-sphering factor present also influence the intermediate crenated shapes between the disk and sphere shapes.

5. Substances from the skin which counteract the activity of the anti-sphering factor may be introduced into a suspension by shaking it against the palm of the hand. Oleic or palmitic acids in a suspension also counteracts the activity of the anti-sphering factor present.

6. Watery ghosts show a peculiar temporary sphering between new slide and cover-glass.

7. The disk-sphere transformation in the absence of anti-sphering factor is reversible. In repeated reversing of the transformation the biconcavities and particular crenations of any one cell always reappear on the same parts of the surface of that cell.

The author is greatly indebted to Dr Henry B. Bull of the Department of Chemistry, North-western University Medical School, and to Dr Eric Ponder of the Biological Laboratory, Cold Spring Harbor, whose criticism and encouragement attributed much toward the success of this research.

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STUDIES ON AMPHIBIAN METAMORPHOSIS

XVII. INFLUENCE OF NON-LIVING ANNULAR TYMPANIC CARTILAGE ON TYMPANIC MEMBRANE FORMATION

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(With Two Plates)

INTRODUCTION

The tympanic membrane of the adult frog is divisible into three histologically distinct parts, viz. an inner epithelial layer, continuous with the mucous membrane of the tympanic cavity, the former being closely applied to a thin lamina propria conforming in shape and area to that of the annular tympanic cartilage to which it is attached at its peripheral borders. The third and outer portion of the membrane consists of modified integument which, externally, appears as a conspicuous brownish coloured area almost circular in shape and slightly indented. The histology of the various layers has been described for only a few anurans. In this regard the reader is referred to Moldenhauer's (1878) work on *R. esculenta* and *R. temporaria*, to that of Tokura (1925) on *R. nigromaculata*, and of the writer's (1928, 1934 *a*) on *R. pipiens*, *R. sylvatica* and *R. clamitans*.

The writer (1928) has previously shown that the formation of the integumentary portion of the membrane, during larval metamorphosis, involves a definite sequence of histological changes in the skin covering the presumptive ear region. In general, this consists of two distinct phases in which certain degenerative changes first occur in the various integumentary layers, particularly the stratum compactum, followed by a developmental stage during which the characteristic structure of the adult membrane[1] is gradually formed. Thus, the entire process may be considered as a dedifferentiation of normal integument into that type peculiar to the tympanic membrane.

The skin of the presumptive ear region cannot be considered as determined to develop into tympanic membrane as contrasted with other integument of the larva, for when it is transplanted, heterotopically, it fails to undergo membrane transformation during metamorphosis. Conversely, however, any integument transplanted to the ear region will transform into tympanic membrane at the proper metamorphic stage. It was finally found that the dedifferentiation of larval skin into that of the tympanic membrane is the result of inductive influences emanating from the developing and underlying annular tympanic cartilage. This conclusion was based on the results of two types of experiments. In the first, the developing annular tympanic cartilage was extirpated at various stages of larval metamorphosis. If removed prior to the initiation of membrane development, no histological membrane changes occurred during metamorphosis. When extirpated after the onset of membrane transformation, the membrane ceased to develop further and in fact tended, in certain cases, to regress towards normal integument. The second type of experiment involved the transplantation of developing annular tympanic cartilage beneath the skin of the side and back. During larval metamorphosis the integument overlying the transplants underwent normal tympanic membrane transformation. It may be noted here that the development of the thickened elastic fibre region of the lamina propria is similarly induced by influences arising from the tip of the developing columella (Helff, 1931).

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[1] The terms "membrane" and "tympanic membrane" as used henceforth in the present paper, refer only to the *integumentary* portion of the tympanic membrane.

The writer (1934 *a*) has furthermore shown that the annular tympanic cartilage continues to exert its inductive influence on membrane formation for some time following complete metamorphosis, but with decreasing intensity. In fact the considerable post-metamorphic enlargement of the tympanic membrane in the growing frog is quite probably due largely, and perhaps entirely, to the corresponding enlargement of the annular tympanic cartilage and its continued inductive effect. Finally, the remarkable intensity of the inductive influence of the developing annular tympanic cartilage has been demonstrated by the writer (1934 *b*, 1937). The fact that fully differentiated dermal plicae integument (transplanted, heteroplastically, over the developing annular tympanic cartilage of the metamorphosing host animal) will undergo complete transformation into tympanic membrane, is convincing evidence of the power of the inductive influence at work.

The nature of the inductive influence or influences present in developing annular tympanic cartilage was of considerable interest to the writer. Since this structure consists entirely of hyaline cartilage, it was thought worth while to determine whether other hyaline cartilage possesses the same inductive effect. For this work (1934 *c*) portions of the quadrate and supra-scapula were removed from newly metamorphosed *R. palustris* frogs and transplanted, autoplastically, beneath the back integument. The results showed that membrane inductive influences of varying intensities were present in such transplants. Usually only slight tympanic membrane transformation occurred in skin over the supra-scapula transplants, while the quadrate implants induced nearly complete membrane development in many cases. This can be readily understood when one considers the fact that the quadrate and the annular tympanic cartilage are quite closely related, embryologically; the latter structure having its origin from the metapterygoid region of the larval quadrate.

Apparently, therefore, tympanic membrane influences are characteristic of hyaline cartilage in general, although the inductive intensity is subject to considerable variation and is particularly strong in the developing annular tympanic cartilage. The fact that actual contact of cartilage and integument is essential before membrane induction will ensue, strongly suggests that the inductive influence is probably chemical in nature. In fact, it was consistently noted that where contact between transplant and skin was strongest, the membrane development was correspondingly accelerated and more pronounced as compared with regions where implant and integument were barely touching one another and the former, therefore, exerting no pressure against the latter.

In the present work an attempt has been made to carry the problem a step further. The question of whether or not non-living annular tympanic cartilage still possesses inductive influences was investigated. Should no membrane induction occur following the transplantation of dead annular tympanic cartilage beneath integument, the results would indicate, although not necessarily so, that the inductive influence is not chemical in nature. However, it is obvious that such a negative result might be explained on the grounds that normally the inductive chemical influence is being constantly produced by the living cartilage in amounts sufficient to initiate and complete membrane development, whereas in the killed cartilage only a limited amount is present which, when used up, cannot be replenished by the dead cartilage cells. If membrane formation was found to be induced by dead annular tympanic cartilage, the probability of the presence of a chemical or secretory influence would be strongly suggested.

MATERIALS AND METHODS

The species utilized for the various transplantations were *R. palustris* and *R. catesbeiana*. Specimens of the former species were collected in the vicinity of Lake Winnisquam, New Hampshire, during the month of July 1938, while those of the latter species were obtained from Silver Lake, New Hampshire, during August of the same year. Usually the animals, when collected, were at or near the metamorphic stage desired for operative work, or if not, soon attained the proper degree of transformation in the laboratory.

The technique of annular tympanic cartilage extirpation has been previously dealt with in some detail by the writer (1928, 1934 *c*) and need not be related here. The methods employed to kill the transplants, however, prior to their implantation beneath back integument, should be described at this time since, as far as the author is aware, the killing of annular tympanic cartilage for subsequent transplantation work has not been done before.

Four killing methods were used, viz. immersion in alcohol, prolonged chloretonization, boiling in water, and desiccation. For the alcohol treatment, the extirpated annular tympanic cartilages were placed in 95% ethyl alcohol for 5 min. in some instances and for 30 min. in others. Cartilages, so treated, become more firm and tough and are consequently easy to transplant. There is no apparent shrinkage in size. Subsequent observation showed that while the transplants had been killed when immersed for but 5 min., they apparently had not been preserved against degeneration as adequately as had those treated for 30 min. Killing by chloretonization involved simply the immersion of transplants in a saturated solution of chloretone (trichloro-tert-butyl alcohol) for approximately 10 min. There is no immediate physical effect on the cartilages and they appear the same as when extirpated. When killed in water, the cartilages were boiled for about 5 min. in either tap or distilled water. This treatment, although not shrinking the transplant caused it to become very soft and white, making its transplantation somewhat difficult. The desiccation treatment involved more care and difficulty as compared with the other methods of killing. Desiccation was quickly, although no doubt not entirely accomplished, by placing the rings of cartilage on a piece of filter paper after which they were exposed to strong direct sunlight for at least an hour. The transplants by the end of this period become hard and brittle and have a tendency to curl up at the periphery. They are also considerably reduced in size. Since they tend to adhere rather firmly to the filter paper, care had to be exercised in removing them to prevent breakage or other injury. It is interesting to note here, however, that following transplantation beneath integument, they imbibe lymph rapidly and return to their normal size and general physical consistency within 5–10 min. Cartilages killed with alcohol or chloretone were always carefully washed in water for at least 10 min. prior to their transplantation. Those killed by means of desiccation or hot water, however, were transplanted without further treatment.

All transplantation was autoplastic and the cartilage was always obtained from the right side. In the case of *R. palustris* animals, the transplants were placed beneath the back integument in the mid-dorsal line just posterior to the eyes. To accomplish this a small transverse incision in the integument was first made quite far posterior on the back. The cut was just large enough to permit of the entrance of the transplant. A small spear-headed scalpel was next inserted through this incision and the back integument carefully loosened anteriorly by cutting the subcutaneous septa or connecting fibres which bind the skin to the underlying musculature. The transplant is now inserted through the cut in the skin and carefully shoved anteriorly to its desired position. Care must be exercised in so doing since the transplant can be easily seriously injured or doubled over on itself. When properly implanted, however, it produces a moderate to a strong bulging of the integument above it by means of which the outline of the transplant can be readily detected externally. Transplantation on *R. catesbeiana* animals was made on the right side beneath the skin of the upper jaw to a point just posterior-ventral to the external nares. The transplants were first inserted through a skin incision ventral to the eyes and then moved anteriorly to the desired position. Even more care is necessary here in loosening the integument under which the cartilage transplant must pass since it is even more firmly attached than is the case of back skin. Transplants in this location, however, maintain an even stronger contact with the overlying integument and usually produce a very pronounced bulging of the latter. This condition was considered of advantage for the purpose of the experiment.

All operated animals were maintained in separate aquaria, the water changed daily, and frequent observations made. Representative specimens were killed in 70% alcohol for photographic purposes. All specimens killed for subsequent histological study were fixed in an aqueous solution of Bouin's fixative. The killing and fixation of *R. palustris* animals was made on the seventeenth to the twenty-ninth day following transplantation, although the great majority were fixed between the twenty-third and twenty-ninth days. *R. catesbeiana* specimens were fixed between he eighteenth and twenty-third days following annular tympanic cartilage transplantation.

The animals, at the time of cartilage transplantation, were in approximately one of two stages of larval involution. Stage A larvae were newly metamorphosed young frogs in which the tail had been entirely resorbed or was represented by a black stump not over 8 mm. (*R. palustris*) or 15 mm. (*R. catesbeiana*) in length. In such individuals the normal tympanic membrane was about half developed, histologically, and had begun to assume a brownish tint as viewed externally (Pl. I, fig. 1). The annular tympanic cartilages extirpated from such individuals were of good size and quite circular in shape. Stage B animals were characterized by a much earlier stage of larval involution in which the tail was little shortened in length but definitely antrophied dorso-ventrally. The forelegs had emerged, however, and the animal was in general undergoing rapid metamorphosis. The annular tympanic cartilage in such individuals was usually little more than semicircular and about half size as compared with those of stage A animals. Since the cartilage had not as yet reached the definitive tympanic membrane region, there were of course no integumentary changes indicative of membrane development.

The following series of experiments were performed:

Series P (A). R. palustris *control series* (22 cases): Annular tympanic cartilage extirpated from *stage* A animals and transplanted, autoplastically, beneath integument of the *back*.

Series P (B). R. palustris *control series* (11 cases): Same as series P (A) except *stage* B animals used.

Series PAB (14 cases): Same as series P (A) except transplants killed by immersion in *alcohol* for 5 min.

Series PAB (B) (8 cases): Same as series P (B) except transplants killed by immersion in *alcohol* for 5 min.

Series PLAB (15 cases): Same as series P (A) except transplants killed by immersion in *alcohol* for 30 min.

Series PLAB (B) (7 cases): Same as series P (B) except transplants killed by immersion in *alcohol* for 30 min.

Series PCB (14 cases): Same as series P (A) except transplants killed by immersion in *saturated chloretone solution* for 10 min.

Series PCB (B) (8 cases): Same as series P (B) except transplants killed by immersion in *saturated chloretone solution* for 10 min.

Series PDB (16 cases): Same as series P (A) except transplants killed by exposure to *strong direct sunlight* for 1 hr.

Series PDB (B) (9 cases): Same as series P (B) except transplants killed by exposure to *strong direct sunlight* for 1 hr.

Series PWB (15 cases): Same as series P (A) except transplants killed by *boiling in water* for 5 min.

Series C (A). R. catesbeiana *control series* (12 cases): Annular tympanic cartilage extirpated from *stage* A animals and transplanted, autoplastically, beneath integument of *upper jaw*.

Series CAN (7 cases): Same as series C (A) except transplants killed by immersion in *alcohol* for 5 min.

Series CLAN (8 cases): Same as series C (A) except transplants killed by immersion in *alcohol* for 30 min.

Series CCN (6 cases): Same as series C (A) except transplants killed by immersion in *saturated chloretone solution* for 10 min.

Series CDN (9 cases): Same as series C (A) except transplants killed by exposure to *strong direct sunlight* for 1 hr.

Series CWN (8 cases): Same as series C (A) except transplants killed by *boiling in water* for 5 min.

RESULTS[1]

R. palustris *transplantations*

(1) *Series P (A) and P (B).*

In both these series the transplants remained large and of normal circular outline, as could be readily ascertained by the shape and degree of bulging produced in the back integument under which they had previously been transplanted (Pl. I, fig. 2). There were no indications, however, that any enlargement or growth of the cartilage implants had occurred even in those cases where fixation of the animal was not made until the twenty-eighth day. The integument above the transplants was usually under strong pressure (series P (A)) due to the presence of the latter, and under slight to moderate extension in series P (B) where the size of the cartilage implants was much less. In a few series P (A) animals, a brownish coloration, indicative of late membrane formation, was attained. Usually, however, the same greenish, black or grey tint was maintained characteristic of the back integument of newly metamorphosed frogs. Parenthetically, it may be stated that the back integument of metamorphosed *R. palustris* larvae exhibits a wide variation in coloration between different individuals. In all cases where a brownish tint was attained and in many where it was not, a lack of pigmentation developed in those areas of the skin directly over the peripheral portions of the transplant. To the eye, such areas appeared more or less grey and translucent. Although the translucency was usually confined to isolated spots over the transplant rim, several instances were noted in which a complete translucent ring developed. The development of such translucency constitutes one of the normal external manifestations of late membrane formation.

A histological study of the various representative cases sectioned, corroborated the external indications as to the integrity of the transplants. There were no signs of cartilage degeneration and this was true not only of the annular tympanic cartilage but also of the columella which had frequently been included in the original implant (Pl. 2, figs. 14, 17). The areas of integument overlying the more central regions of the transplants gave evidence of pronounced glandular degeneration and correlated reduction in thickness of the stratum spongiosum. The stratum compactum was also undergoing rapid dissociation and fibril degeneration. In several cases there was evidence of the fusion of fibrils within a dissociated bundle. The epidermis in the more central regions was, however, practically unchanged. The integument above and in strong contact with the periphery of the transplant exhibited an even more advanced stage of membrane formation. Here, the epidermis was thinner and corresponded closely to that of normal fully developed tympanic membrane, while the stratum spongiosum was frequently entirely lacking or much reduced in thickness coupled with total glandular degeneration. Of particular interest, in many cases, was the development of the double set of fibrils in the stratum compactum which is characteristic of fully formed tympanic membrane. As previously described by Tokura (1925) for *R. nigromaculata* and by the writer (1928, 1934 *a*) for *R. pipiens*, *R. sylvatica* and *R. clamitans*, these consisted of an inner thick region of horizontal fibrils and an outer region of smaller fibres running vertically, which cross with those of the inner region to form a complex network. When it is added that the subcutaneous connective tissue was frequently entirely absent in this region, the conclusion can be drawn that complete membrane development had been induced (Pl. II, fig. 17). It should also be noted that where the columella had been included in the original transplant, the lamina propria overlying the former was usually greatly thickened and composed chiefly of yellow elastic fibres; demonstrated by staining with Weigert's resorcin-fuchsin (Pl. II, fig. 14). In general, it can be stated that membrane induction was somewhat more pronounced in series P (B) than in series P (A) animals.

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[1] Inasmuch as two or more series usually exhibited practically the same reactions as regards the degree of tympanic membrane induction, they will be discussed together in order to eliminate, as far as possible, needless repetition of results.

(2) *Series PAB, PAB (B), PLAB, PCB and PWB.*

Although the original cartilage transplants of these series were of the same size as those of the comparable control series P (A) and P (B), considerable degeneration ensued following their transplantation. This was evidenced by the slight bulging of the overlying integument at the time the animals were fixed and also by a study of the histological sections. The degeneration was particularly marked in series PAB, PAB (B) and PWB, in which many cases occurred involving no external signs of integumentary bulging indicative of the persistence of an underlying transplant. Sections revealed, however, that a remnant of the cartilage implant always remained. In a few cases the transplants underwent only moderate degeneration and the integument above consequently remained under considerable pressure (Pl. I, fig. 3). The transplants of series PLAB and PCB, although undergoing post-operative degeneration to varying degrees, were in general better preserved. In fact several cases were observed (Pl. I, fig. 4) in which, apparently, no diminution in size had taken place. There were only a few instances in which distinct macroscopic signs of tympanic membrane formation could be detected in the integument overlying the transplants. These occurred in series PLAB and consisted chiefly of coloration changes resulting in the attainment of a brownish tint in two instances. Other than these two cases, there were no other examples of macroscopic signs of membrane formation in any of the five series under discussion.

A histological examination of the transplants revealed decided cartilage degeneration, especially in series PAB and PWB. In series PAB the cartilage cells appeared to be greatly enlarged and vacuolated, while the matrix had decreased in amount (Pl. II, fig. 13). Series PWB transplant cartilage resembled that of series PAB except that the cells usually appeared to be polyhedral in shape and apparently somewhat collapsed. This may very well be, however, simply a later stage of degeneration as represented by the enlarged vacuolated condition. In series PLAB the matrix was very little decreased in amount, while the cartilage cells were only slightly enlarged. Series PCB transplants appeared to be particularly resistant to degeneration and there were many cases in which the cartilage had remained practically the same as when originally implanted.

The histological changes in the integument covering and in contact with the transplants were of that type characteristic of the first or degenerative phase of tympanic membrane formation. These included a general thinning of all layers together with the partial to total degeneration of glandular elements in the stratum spongiosum. The most pronounced changes were observed in series PAB (B), while least degeneration was recorded for series PWB. In all five series, however, the integumentary changes over the periphery of the cartilage transplants were more accentuated as compared with those prevalent in the more central areas (Pl. II, fig. 18). Finally, it should be noted that there were no instances in which the later developmental phase of membrane formation was exhibited.

(3) *Series PLAB (B), PCB (B), PDB and PDB (B).*

The degree of integumentary elevation, indicative roughly of transplant integrity in these series, may be briefly summarized as follows. There were no instances of bulging in series PCB (B), only three in series PDB (B), and but two in series PLAB (B). Individuals of series PDB, however, always showed some degree of integumentary bulging, which in some cases was very pronounced. Usually, the size and shape of the elevation closely approximated that which was produced at the time of the original transplantation, suggesting little if any degeneration of the transplant. Although two cases of integumentary translucency developed in series PDB, there were no other external signs of membrane formation in evidence in these individuals or of any description in the remaining thirty-eight cases of the four series in question.

Histological sections revealed a variety of pictures as regards the condition of the transplants. All cartilages of series PDB (Pl. II, fig. 15) and five of series PDB (B) appeared quite normal. The matrix was of average amount and had taken a natural light blue stain while the cartilage cells were very little, if any, enlarged. Four of series PDB (B) were of unusual interest in that the peripheral portions of the transplants were very evidently undergoing rapid degeneration and dissociation. In this same location were present scattered, deep blue staining, solid masses having the appearance of early bone formation. Occasional irregularly shaped structures, which may have been degenerating cartilage cells, were found imbedded in these masses. The condition of series PLAB (B) and PCB (B) transplants was even more striking for here usually the entire transplant had been obliterated, at least as far as their original cartilaginous make-up was concerned. In their place occurred large irregularly shaped masses of the same texture and staining reaction as previously noted for the four series PDB (B) transplants cited above. The mass, although usually maintaining continuity between its various parts, contained large cavities, while the peripheral portions were characterized by trabecular-like projections and deep pockets; the latter filled with loose connective tissue and connective tissue cells (Pl. II, fig. 16). Somewhat similar cases were encountered in series PWB except that the degenerating cartilage cells (?) were more numerous (Pl. II, fig. 18).

The microscopic appearance of the integument overlying the transplant region in these four series gave even more evidence of tympanic membrane transformation than previously described for the other *R. palustris* experimental series. In addition to a generalized thinning of all integumentary layers, considerable glandular degeneration occurred resulting frequently in their complete absence where the transplant rim was in strong contact with the skin (Pl. II, fig. 15). It was furthermore noted that certain areas of the stratum compactum were dissociating and undergoing degeneration. The development of a double set of stratum compactum fibres (typical of fully formed tympanic membrane and as noted in certain cases of the control series P (A) and P (B)) was not observed.

R. catesbeiana *transplantations*

(1) *Series C (A).*

The transplants of all series C (A) animals maintained moderate to strong pressure against the skin of the upper jaw until such time as the young frogs were fixed for histological study. This resulted in a pronounced elevation of the integument, the height and diameter of which served to indicate that while the cartilage had not increased in size, it had obviously undergone no decided degeneration (Pl. I, fig. 5). Histological examination, moreover, showed that the normal cytological condition of both the annular tympanic cartilage (Pl. I, fig. 9) and the columella (Pl. I, fig. 10) had remained unchanged.

The external appearance of the integument overlying the transplants underwent changes indicative of membrane formation. In three cases this consisted of a coloration change in which a circular band of skin in contact with the rim of the transplant had turned to a brownish shade. The more central areas of the integument, however, still remained greenish in coloration similar to that of the adjacent skin of the jaw. This condition is not abnormal, since in the normal development of the tympanic membrane the integumentary transformations involved always begin at the periphery and progress towards the centre. In the remaining nine cases, however, development had proceeded to the extent where the entire circular area over the transplant was of a nearly uniform brownish tint. Considerable translucency also developed in these individuals. The general appearance compared quite favourably with that of the normal left-side tympanic membrane of the same animal (compare Pl. I, figs. 5, 7).

Sections made of the integument covering the transplants showed that although no decrease in thickness usually occurred in the more central areas, that associated with the transplant rim was invariably much thinner as the result of degenerative changes (Pl. I, fig. 9). The epidermis of this region was slightly decreased in thickness, which was also true of the stratum spongiosum. The failure of the latter layer to degenerate completely was probably due to the presence of many large mucous glands, which are characteristic of the jaw integument in *R. catesbeiana* much more so than is the skin of the normal tympanic membrane region. It was noted, however, that the nearer to the rim of the transplant one approached, the smaller the glands became, while many were apparently undergoing degeneration. The stratum compactum gave more clear-cut evidence of membrane transformation. In addition to the loss of its convoluted appearance coupled with the partial degeneration of its constituent fibres, there was evidence of the development of the double set of fibres typical of fully formed tympanic membrane. Mention should also be made of the fact that where the columella had been incorporated in the transplant the lamina propria covering its tip was greatly thickened and composed almost entirely of yellow elastic fibres (Pl. I, fig. 10).

(2) *Series CAN, CLAN, CCN, CDN and CWN.*

The integrity of the transplants in the *R. catesbeiana* experimental series, as evidenced by the degree of integumentary bulging, was subject to considerable variation. There were but four cases in which the cartilage implants had degenerated to the extent of no longer producing an elevation of the adjacent skin. There were two each of these in series CAN and CDN. In the remaining thirty-four individuals, however, slight to strong bulging of the integument was maintained, the majority producing a moderate elevation of the skin. Frequently, the elevated area was not completely circular in outline, indicating either a mechanical doubling over of the transplant, due to the difficulty of proper implantation, or (as later shown by histological examination) to a more rapid degeneration of one region of the cartilage as compared with another (Pl. I, fig. 6). It is of interest to compare this condition with the appearance of the half-formed membrane in the ear region on the same side of the animal (Pl. I, fig. 8). At the time of annular tympanic cartilage extirpation for transplantation further anterior, the integument of this region had evidenced distinct macroscopic signs of membrane formation which have persisted for 19 days in this case. In the absence of the cartilage, however, the area affected has not increased in size as can be determined by comparison with the left-side normal membrane of the same individual (Pl. I, fig. 7). There were only two series in which macroscopic changes occurred in the integument, overlying the transplants, indicative of membrane formation. In series CCN, two instances occurred in which a slight translucency was evident, particularly in those areas in contact with the periphery of the transplant. In series CWN, four cases were noted in which not only translucency developed but a distinct brownish coloration was apparent.

Sections made through the transplant region showed that in all instances varying degrees of cartilage degeneration had taken place. Since the histological icture differed, considerably, between the various experimental series, a brief escription for each is necessary. Series CAN transplants usually showed a normal mount of matrix but with the cartilage cells collapsed and misshapen. In series LAN, the matrix was somewhat reduced in proportion, while the cellular elements ere generally larger, ovoidal in shape, and definitely vacuolated (Pl. II, fig. 20). eries CCN transplants were usually degenerated more peripherally than centrally ith cartilage cells slightly vacuolated (Pl. I, fig. 11). The transplants of series CDN ere always undergoing rapid degeneration and as a result were greatly reduced in ze. Furthermore, they were characterized by a minimum of matrix and large, acuolated cells (Pl. I, fig. 12). Lastly, series CWN transplants were peculiar in hat although the cartilage cells were almost invariably misshapen with large nuclei, hey were nevertheless usually small in size and not vacuolated (Pl. II, fig. 19).

The integument over series CAN, CLAN, CCN and CDN transplants usually emained quite normal, histologically (Pl. I, figs. 11, 12; Pl. II, fig. 20). There was o appreciable thinning of the various skin layers while the large mucous and oison glands in the stratum spongiosum appeared quite normal as compared with hose in adjacent integument. A few cases in series CCN were noted, however, in hich slight glandular degeneration was apparent. In contrast to the above series, he integument in contact with series CWN transplants showed slight to pronounced igns of degeneration. This usually resulted in a definite thinning of the skin, specially at the expense of the stratum spongiosum and stratum compactum layers. n the former, pronounced glandular degeneration was usually seen, while the atter layer was characterized by dissociation and degeneration of its constituent bre bundles (Pl. II, fig. 19). The development of fully formed tympanic membrane tratum compactum fibres was not observed, however. Mention should also be made f the fact that scattered ossifying (?) masses were frequently seen in series CCN, DN and CWN in the loose connective tissue surrounding the transplants (Pl. I, gs. 11, 12; Pl. II, fig. 19). No generalized or central ossification of the cartilage as observable.

DISCUSSION

Previous work of the writer has demonstrated the potentiality of side and back ntegument to undergo tympanic membrane transformation when in contact with ransplanted annular tympanic cartilage. The results of the present work on *. catesbeiana* lead one to conclude that jaw integument must be added to this list. he latter, however, is apparently not as susceptible to such transformation as is rue of the former two integuments. This is quite probably due to the presence of umerous and abnormally large glands in the stratum compactum layer, which nust first undergo degeneration before the developmental phases of membrane ormation can take place.

The results of the present work furthermore demonstrate and emphasize the oint that membrane induction is essentially a *contact* phenomenon. The greater he area of contact with the cartilage and the stronger the pressure of the latter against the integument, the more certain and rapid will be the membrane formatio Just how the pressure factor operates is somewhat problematic. Inasmuch a integument under the strong pressure of an implanted glass bead (Helff, 1926) wi undergo complete degeneration and even perforate, it is logical to inquire whethe the degenerative phase of membrane formation might be due to this factor alon Although a few isolated cases of minute perforations occurred in the skin over th transplants, subsequent histological study of these instances and of all othe integument sectioned showed that the sequence of degenerative changes that ha occurred were typical of tympanic membrane formation and not of the generalize type characteristic of "pressure atrophy". It seems more likely, therefore, tha strong contact involving pressure functions chiefly to insure a more rapid trans ference of the chemical or other inductive factors in the annular tympanic cartilag to the integument.

It is of interest to inquire whether the degenerative changes which occurred i the integument of the experimental series were due simply to contact with a dea degenerating mass of tissue (the transplant) or represented the normal first phas of tympanic membrane formation. That the latter conception was no doubt th case, is borne out by a consideration of the following points: In the first place, th degeneration which occurred did not involve all integumentary layers at the sam time or with a uniform rate, which is apt to be the case where disintegration caused solely under the influence of adjacent decomposing tissue. As previous stated, the various degenerative changes occurred at varying rates and in a sequen typical of normal membrane formation. Secondly, complete degeneration of th overlying integument did not occur but only to that extent normally characterist of membrane formation. Finally, there were many instances in the *R. catesbeian* series in which no integumentary dissociation occurred at all in spite of the fact th the transplants were undergoing pronounced degeneration.

An analysis and comparison of the results of the fourteen experimental seri (exclusive of the control series) reported in the present paper, lead one to conclu that there exists no consistent correlation between the type of killing meth employed for the cartilage transplants and the latter's subsequent average degree degeneration. For example, transplants immersed in alcohol for 30 min., underwe practically no degeneration in series PLAB, moderate degeneration in series CLA and excessive degeneration in series PLAB (B). The only definite correlation note in this regard concerned those transplants killed by chloretone solutions, in whic moderate cartilage degeneration occurred in both stages A and B *R. palustris* seri and the *R. catesbeiana* series. Within any one series, however, the results we usually more consistent, although instances of rather wide variation were sometim recorded. A still further analysis was made to determine whether a correlation w evident between the degree of transplant degeneration and the extent of tympan membrane induction. Although instances of non-correlation were found, it may said that in general the *greater* the cartilage degeneration, the *less* the membra induction in the adjacent integument. This was true for both stages A and *R. palustris* series and the *R. catesbeiana* series.

In considering the various cytological changes characteristic of membrane ormation which occurred in the integument, the peculiar and transitory hyperrophy of the basal layer of epidermal cells (described by the writer, 1928, for *R. pipiens*) was not observed in either *R. palustris* or *R. catesbeiana*. It is possible, of course, that this particular degenerative feature is not an essential feature of membrane formation in all forms and is lacking in the two species in question. Again, it may be that in the present instances, it occupies a different position in the sequence of histological changes and normally occurs at a later stage of membrane ormation than was attained in any of the transplant series. This is considered rather unlikely, however, since such a late development as typical membrane stratum compactum fibres was obtained quite frequently in the control series of both species. What seems more probable is that the transforming integument was not fixed for sectioning at the right stage of membrane formation. In this regard it may be stated that the phenomenon of epidermal cellular hypertrophy occupies a very brief period of time and its presence in *R. pipiens* was only detected after sectioning nteguments in many closely related stages of membrane transformation.

The development of a double set of fibres in the stratum compactum constitutes one of the last steps in integumentary transformation and hence practically characterizes complete membrane formation. It is of interest and importance, therefore, to inquire why some of the control animals and all of the experimental individuals failed to attain this degree of development. As for the stage A control animals, it can only be suggested that when the annular tympanic cartilage was removed for transplantation, the normal tympanic membrane was approximately already half-formed. Hence, considerable of the total inductive power of the cartilage had already been expended. Furthermore, when transplanted to its new location, the cartilage, although showing no signs of degeneration, failed to increase in size and no doubt became less potent as regards membrane induction. Stage B transplants, although in a more active stage of membrane induction, no doubt failed to induce complete integumentary transformation for the same reason. It will be recalled in this regard, however, that membrane formation in the stage B control series was usually more advanced than was true of the stage A control series.

The failure of the experimental series to develop membranes to as full an extent as the control series, calls for another explanation. In the first place, the killing of the cartilage prior to its implantation, quite probably made impossible the subsequent secretion or formation of additional inductive substances. Thus, as the inductive substances present were gradually utilized, there came a time when the supply was exhausted, resulting in a cessation of membrane induction. Still another contributory factor should be mentioned in this regard. As has previously been mentioned, most transplants of the experimental series underwent disintegration and in general the greater the degree of cartilage degeneration, the less the extent of membrane formation. This would seem to imply, therefore, that during the disintegration of the cartilage, substances are released or formed which are capable of neutralizing the inductive substances or of counteracting their effect on the integument.

The results of experiments described in the present paper lead the writer t believe that the integumentary portion of the anuran tympanic membrane developed largely, and possibly entirely, through the action of an inductiv substance or substances produced by the developing annular tympanic cartilag Although the exact nature of these substances is still problematic, it seems qui logical to predict that they will ultimately be shown to be either direct secreto products of the cartilage cells, products arising from the ageing of the matrix o other chemical changes occurring within it, or perhaps a combination of both. I any event, a *chemical* inductor is, obviously, strongly indicated. There can be n question of the fact that the inductive substances cannot become functional unt transmitted to the larval integument of the ear region through contact of th annular tympanic cartilage. Furthermore, continued contact with the cartilag during the entire developmental stage of the membrane, is essential if complet integumentary transformation into tympanic membrane is to occur. That this is case of complete dependence is further emphasized by the fact that even enlargement of the membrane during the post-metamorphic life of the frog is entirel dependent on the growth and corresponding enlargement of the annular tympani cartilage with which it must maintain contact (Helff, 1934 *a*). There is som evidence serving to indicate that the gradual thinning of the integumentary layer (particularly the epidermis), during membrane formation, may be partially due t the action of increased tension within these layers; the latter being due to pressur exerted by the developing annular tympanic cartilage.

SUMMARY AND CONCLUSIONS

Previous work by the writer has shown that normal development of the tympani membrane in *R. pipiens* is due to inductive influences of the annular tympani cartilage. The present work concerns the nature of the developmental influence specifically as regards whether or not non-living cartilage still possesses inductiv properties. The experiments performed and results obtained were as follows:

Controls consisted of autoplastic transplantation of annular tympanic cartilag beneath back integument of metamorphosed and involuting *R. palustris* larvae. I general, integumentary thinning, coupled with partial to total glandular degeneration, occurred. This is typical of partial membrane formation.

Similar transplantations were made involving cartilage killed in ethyl alcohol saturated chloretone solution, and boiling water, or by desiccation in strong sunlight. The results differed considerably depending on the type of killing treatmen employed and the degree of subsequent transplant degeneration. Partial membran formation was usually induced, however, in the overlying back integument. Simila series were run with half-metamorphosed larvae. In general, membrane inductio was even more pronounced.

The same experiments were performed on *R. catesbeiana* larvae. Transplantation, however, was made beneath the skin of the upper jaw, posterior-ventral to th external nares. Wide variations in results were observed. No membrane change occurred in some cases, while in others considerable transformation was induced.

It is concluded that dead annular tympanic cartilage still retains inductive influences capable of initiating varying degrees of tympanic membrane formation in integument. In general, the greater the degree of cartilage degeneration, the less the extent of membrane formation.

The results are discussed in general and the conclusion reached that the inductive influences arising in the developing annular tympanic cartilage of the anuran are quite probably chemical secretions or excretions. These, when transmitted to the integument of the ear region during metamorphosis, serve to induce a dedifferentiation of the larval skin into that type characteristic of adult tympanic membrane.

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EXPLANATION OF PLATES I AND II

Plate I

Fig. 1. Macroscopic appearance of half-developed *R. palustris* tympanic membrane of stage A animals when annular tympanic cartilage was extirpated for transplantation. Membrane beginning to assume a brownish tint. × 12.

Fig. 2. Macroscopic appearance of back integument 26 days after transplantation of normal annular tympanic cartilage beneath it. Pronounced elevation of skin with peripheral translucency developing. (Case P (A) 8.) × 8·5.

Fig. 3. Macroscopic appearance of back integument 27 days after transplantation of dead annular tympanic cartilage beneath it. (Cartilage killed by immersion in alcohol for 5 min.) Pronounced elevation and generalized paling of skin over transplant. (Case PAB 4.) × 7·5.

Fig. 4. Macroscopic appearance of back integument 26 days after transplantation of dead annular tympanic cartilage beneath it. (Cartilage killed with chloretone.) Pronounced elevation of skin indicating well-preserved transplant, but no distinct colour changes in the former. (Case PCB 8.) × 7·5.

Fig. 5. Macroscopic appearance of jaw integument 19 days after transplantation of normal annular tympanic cartilage beneath it. Pronounced elevation of skin with generalized brownish coloration and peripheral translucency. (Case C (A) 6.) × 6.

Fig. 6. Macroscopic appearance of jaw integument 19 days after transplantation of dead annular tympanic cartilage beneath it. (Cartilage killed by immersion in alcohol for 30 min.) Moderate irregularly shaped elevation of skin resulting from partial degeneration of transplant. There are no distinct colour changes apparent. (Case CLAN 1.) × 6.

Fig. 7. Macroscopic appearance of normally developing tympanic membrane of the *left* side 19 days after transplantation of the *right* side annular tympanic cartilage of the same individual beneath jaw integument. The large size, brownish coloration, and translucency of the membrane are proofs of the continued growth and active inductive power of the annular tympanic cartilage beneath. (Case CLAN 1.) × 6.

Fig. 8. Macroscopic appearance of normally developed half-formed tympanic membrane in right-side ear region of the same individual cited in figs. 6 and 7, 19 days after the extirpation of the annular tympanic cartilage. Comparison with fig. 7, shows that no further membrane development occurred following the removal of the underlying cartilage. (Case CLAN 1.) × 6.

Fig. 9. Histological section through jaw integument overlying transplant *rim* 19 days after transplantation of the latter. The various layers have undergone definite thinning and degeneration notably the stratum compactum. Note the persistence of the large glands in the stratum spongiosum and the normal appearance of the cartilage. (Case C (A) 5.) × 142.

Fig. 10. Histological section through jaw integument overlying *columella* portion of transplant 19 days after transplantation of the latter. Same individual as cited in fig. 9. Although only slight thinning has occurred in the integument, the lamina propria lying between it and the columella i greatly thickened and composed chiefly of yellow elastic fibres. Note normal appearance of th cartilage. (Case C (A) 5.) × 62.

Fig. 11. Histological section through jaw integument and transplant 19 days after transplantation o dead annular tympanic cartilage. (Cartilage killed with chloretone.) The integument appears normall although the cartilage is degenerating. Note dark-staining ossifying (?) masses above and particularl below the transplant. (Case CCN 3.) × 82.

Fig. 12. Histological section through jaw integument and transplant 18 days after transplantation o dead annular tympanic cartilage. (Cartilage killed by desiccation.) The integument appears normall but the cartilage is greatly reduced in size. Note large vacuolated cells in the transplant and th presence of dark-staining ossifying (?) masses along either side of it. (Case CDN 2.) × 82.

PLATE II

Fig. 13. Histological section through back integument and transplant 28 days after transplantation o dead annular tympanic cartilage. (Cartilage killed by immersion in alcohol for 5 min.) Definite thinning of all integumentary layers is seen, coupled with partial to total glandular degeneration The transplant is degenerating rapidly as evidenced by the extremely large and vacuolated cartilage cells. (Case PAB 5.) × 142.

Fig. 14. Histological section through back integument overlying *columella* portion of transplant 23 days after transplantation of the latter. The integumentary layers are degenerating and glandular disintegration is nearly complete. The lamina propria lying between the integument and the transplant is greatly thickened and composed chiefly of yellow elastic fibres. Note normal appearance o the cartilage. (Case P (A) 17.) × 142.

Fig. 15. Histological section through back integument overlying transplant *rim* 26 days after transplantation of dead annular tympanic cartilage. (Cartilage killed by desiccation.) All integumentary layers above the transplant are thin and degenerating, while glandular disintegration is complete. The cartilage has remained normal. (Case PDB 2.) × 142.

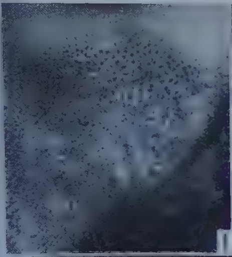
Fig. 16. Histological section through back integument and transplant 24 days after transplantation o dead annular tympanic cartilage. (Cartilage killed by immersion in alcohol for 30 min.) All integumentary layers are thin and degenerating, while glandular disintegration is nearly complete. The transplant has either been replaced by, or transformed into, a dark staining, irregularly shaped, ossifying (?) mass in which occasional, degenerating cartilage cells are seen. (Case PLAB (B) 2.) × 142.

Fig. 17. Histological section through back integument overlying transplant *rim* 23 days after transplantation of the annular tympanic cartilage. Same individual as cited in fig. 14. Practically complete dedifferentiation of the integument into that type characteristic of adult tympanic membrane. Note thinness of all layers, complete degeneration of glands, and absence of a subcutaneous connective tissue. The cartilage has remained normal. (Case P (A) 17.) × 142.

Fig. 18. Histological section through back integument and transplant 25 days after transplantation of dead annular tympanic cartilage. (Cartilage killed with boiling water.) Thinning of integumentary layers and glandular degeneration, especially where transplant is maintaining pressure against the former. The transplant has either been replaced by, or transformed into, a dark-staining, irregularly shaped, ossifying (?) mass in which many large vacuolated objects (degenerating cartilage cells?) are seen. (Case PWB 6.) × 142.

Fig. 19. Histological section through jaw integument and transplant 19 days after transplantation of dead annular tympanic cartilage. (Cartilage killed by boiling water.) Although the epidermis is but slightly thinned, pronounced degeneration of all other integumentary layers is evident. Glandular disintegration is especially marked. The transplant, also degenerating, contains many small, misshapen, non-vacuolated cartilage cells with large nuclei. Note the numerous, dark-staining, ossifying (?) masses, particularly between the transplant and the integument. (Case CWN 3.) × 82.

Fig. 20. Histological section through jaw integument and transplant 19 days after transplantation of dead annular tympanic cartilage. (Cartilage killed by immersion in alcohol for 30 min.) The integument appears quite normal with many large mucous glands; characteristic of jaw integument. The transplant matrix is reduced in proportion to the cartilage cells, while the latter appear to be enlarged and vacuolated. (Case CLAN 2.) × 82.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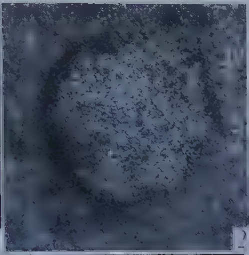
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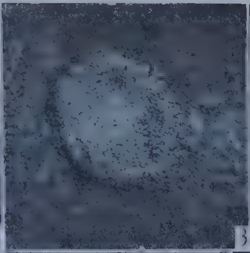
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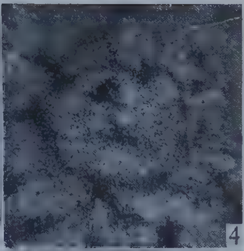
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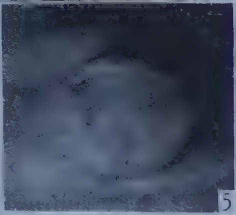
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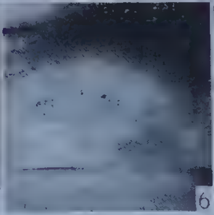
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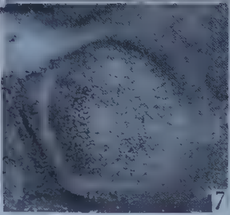
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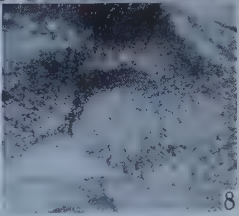
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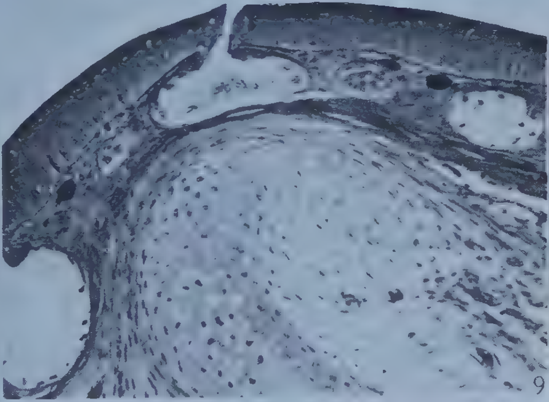
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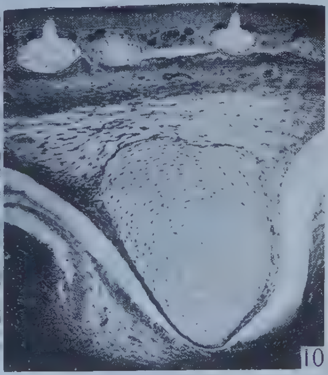
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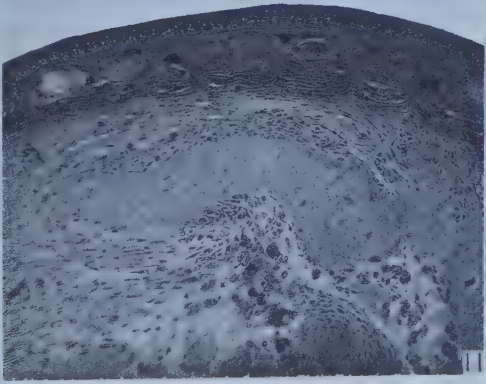
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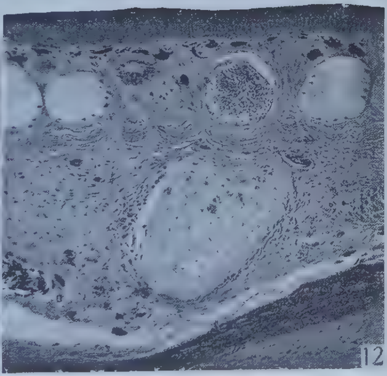
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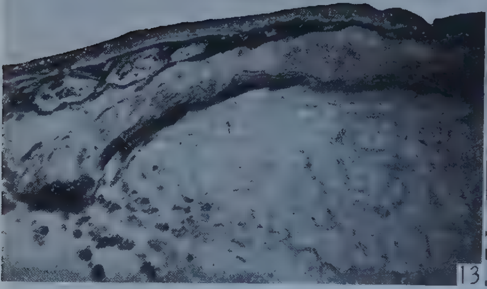
HELFF—STUDIES ON AMPHIBIAN METAMORPHOSIS (pp. 45-60)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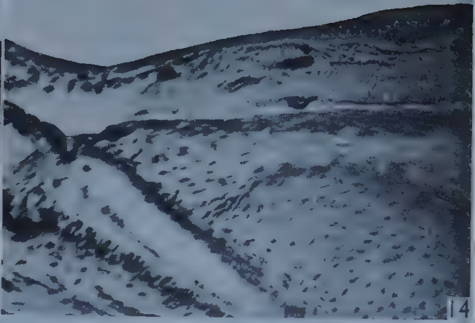
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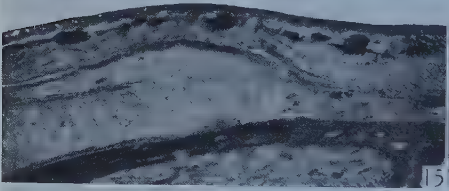
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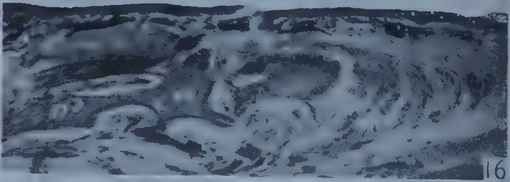
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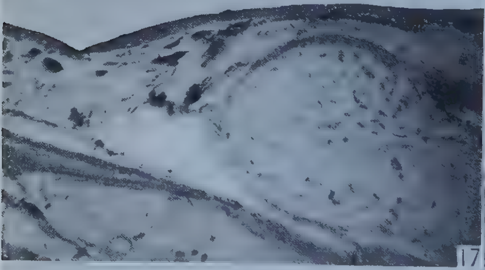
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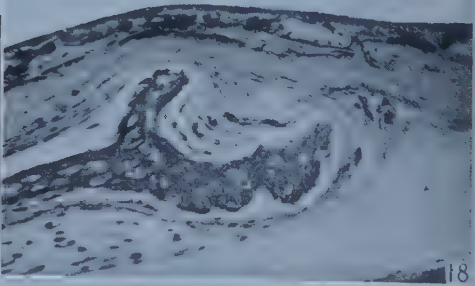
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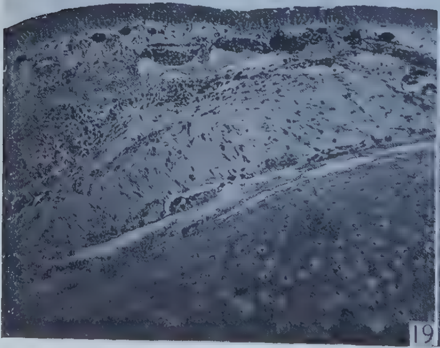
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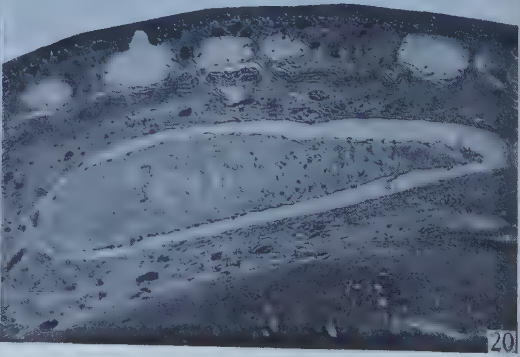
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HELFF—STUDIES ON AMPHIBIAN METAMORPHOSIS (pp. 45–60)

FACTORS INFLUENCING FACILITATION IN ACTINOZOA. THE ACTION OF CERTAIN IONS

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(With Six Text-figures)

In at least some of the muscles in Coelenterates the size of response is controlled by the neuromuscular junction. The sphincter in *Calliactis* and the longitudinal mesenteric retractor muscles of *Metridium senile* are served by part of the nerve network which acts as a single excitable system. The response is not graded through a large number of separate motor units. A single stimulus applied to this nerve net is ineffective, or only causes a trivial response; but it leaves behind it an effect which facilitates the transmission of a second impulse to the muscle. Each impulse in a series facilitates the transmission of excitation to more and more muscle fibres. After each stimulus the facilitating effect gradually dies away (Pantin, 1935*b*). In a short discussion, the relation of this "facilitation" process to other cases of junctional transmission was considered (Pantin, 1937). The process by which excitation is only effective if one stimulus follows its predecessor before the period of facilitation has departed, is analogous to the condition of vertebrate skeletal muscle during partial curarization. But at present the nature of the facilitation process is still unknown. A study of the action of ions and other substances throws considerable light upon its nature, and it is the object of this paper to describe and discuss these effects.

The anemones, *Calliactis parasitica* and *Metridium senile*, were exposed to mixtures of sea water with various substances or with isotonic solutions of the chlorides of various cations. The contractions of the muscles were recorded in response to a series of condenser shocks applied to the column of the animal (Pantin, 1935*a*). The shocks were sent in through Ag-AgCl electrodes.

Fig. 1*a* shows a response of the marginal sphincter when *Calliactis* is given a series of four shocks at frequencies of 2 per sec., 1 per sec. and 1 every 2 sec. As in all normal contractions, there is no response to the first stimulus and the response increases considerably as the frequency rises. In all the experiments that follow, similar series of stimuli at these frequencies were given as controls and to illustrate the effect of various substances. The experiments were conducted at room temperature, 18–21° C.

CARBON DIOXIDE

Saturated solutions of carbon dioxide in sea water were made. The first effect c immersing the anemone in such a solution is to cause protrusion of the mouth an oral disk. Slow peristaltic contractions of the column may then follow. Thes effects soon pass off and within 10 min. the characteristic anaesthetic action c CO_2 becomes apparent and continues gradually to increase, being fairly complete i 1–2 hr. (Fig. 1). On return to natural sea water recovery is complete in less tha 1 hr.

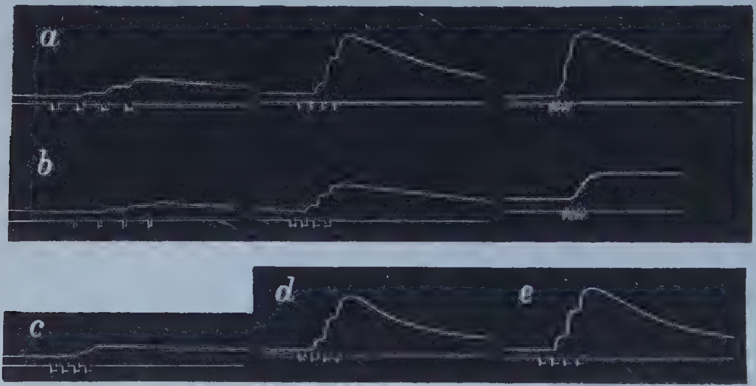

Fig. 1. Responses of the sphincter of *Calliactis* to stimuli at 1 in 2 sec., 1 in 1 sec., and 1 in 0·5 sec. in (*a*) natural sea water, (*b*) after 10 min. in sea water saturated with CO_2 (pH 6·5), (*c*) after 60 min. in CO_2 sea water (stimuli 1 in 1 sec.), (*d*) 70 min. after return to sea water following 70 min. in CO_2 se water (stimuli 1 in 1 sec.), (*e*) effect of 70 min. in aerated sea water brought to pH 6·5 with HCl.

When the sphincter no longer responds to stimulation of the anemone on the column, direct stimulation of the muscle still produces a good response. Since the muscle is not itself incapable of contraction the failure to respond must be due either to failure of the nerve net or of the neuromuscular junction. But failure o response develops as a gradual reduction of the size of the contraction, not as its abrupt abolition. Hence the nervous impulses set up by the stimuli are still reaching the muscle as in the normal animal, though the response to them is less. We may conclude that the primary action of CO_2 is on the neuromuscular junction by depression of facilitation. Mayer (1916) showed that in *Cassiopeia* carbon dioxide can prevent muscular response without affecting nervous conduction.

Though the prime effect of carbon dioxide is upon the junction, it has some direct effect on both the nerve net and the muscle. The threshold of excitation steadily rises, reaching two or three times the normal at the point when indirect response of the muscle fails. The effect on the muscle is a slowing of the response, particularly of the relaxation. This is similar to the effect described by Bozler (1931) on the buccal retractor of *Helix*. These effects appear to be due specifically to carbon dioxide. Aerated sea water brought to the same pH, 6·5, has no obvious action (Fig. 1 *e*).

The depression of facilitation by carbon dioxide is greatest at the higher frequencies. The muscular response cannot be made as good as a normal one by raising the frequency of stimulation, whereas the maximal interval between stimuli which will just enable a response to take place is not greatly reduced. The depression is thus not due to an increased rate of decay of facilitation but to a reduced responsiveness of the muscle. The effect is strikingly different from that of a rise in temperature when the animal is in sea water. In this case the responses are quite normal, but facilitation decays at a much faster rate so that the frequency at which all reactions take place is higher (Hall & Pantin, 1937).

Even under profound anaesthesia with carbon dioxide the first contraction to a series of stimuli still takes place on the second stimulus as in the normal animal. But after prolonged exposure several stimuli may be required before the muscle responds. A similar effect occurs under excess magnesium and it will be shown that it is due to breakdown of conducting tracts in the nerve net leading to "interneural facilitation", and not to modification of the neuromuscular junction.

MAGNESIUM

Excess magnesium depresses the responses reversibly. Within less than a minute superficial sense organs are anaesthetized, especially in the tentacles. The subsequent effects on the neuromuscular system are both slower to appear and slower to show recovery, than those of carbon dioxide. This is perhaps because of difficulty of diffusion into the thick mesogloea. Magnesium slowly lowers the electric excitability of the nerve net. A mixture of 50 % sea water with 50 % 0·4 M $MgCl_2$ (pH 8·0) causes little change in the threshold for about half an hour, but then this rises steadily though it only reaches about twice the normal value when anaesthetization becomes complete.

The greatest effect of magnesium is on facilitation (Fig. 2). In the mixture just referred to the facilitated response of the sphincter in *Calliactis* begins to fall off progressively within 5 or 10 min. The individual contractions themselves do not become slower as under the influence of carbon dioxide; indeed they often become sharper with more rapid relaxation. But as under carbon dioxide the size of the responses becomes steadily smaller over a period of an hour or so and finally vanishes. This depression of the response is again greatest at the highest frequencies of excitation so that shortening of the interval between the stimuli does not enable the contractions to attain normal size (Fig. 2*d*). Although magnesium reduces the size of all contractions, these first become visible at about the same frequency of stimulation as in normal conditions. These facts indicate that magnesium, like carbon dioxide, depresses facilitation but does not increase its rate of decay. Until the response has almost disappeared the rule that the first contraction appears on the second stimulus is strictly adhered to. At this stage the sphincter responds well to direct stimulation, and just as with carbon dioxide, the effect of magnesium must be primarily a depression of facilitation at the neuromuscular junction. This agrees with the experiments of Mayer (1916) on conduction in the subumbrella net of

Cassiopeia, and of Parker (1932) on conduction in the filaments of *Physalia*. In both these cases magnesium can locally abolish the response of the muscle to indirect stimulation without the abolition of conduction through the nerve net. Katz (1936) has shown in the skeletal muscle of *Carcinus maenas* that magnesium depresses facilitation at the neuromuscular junction.

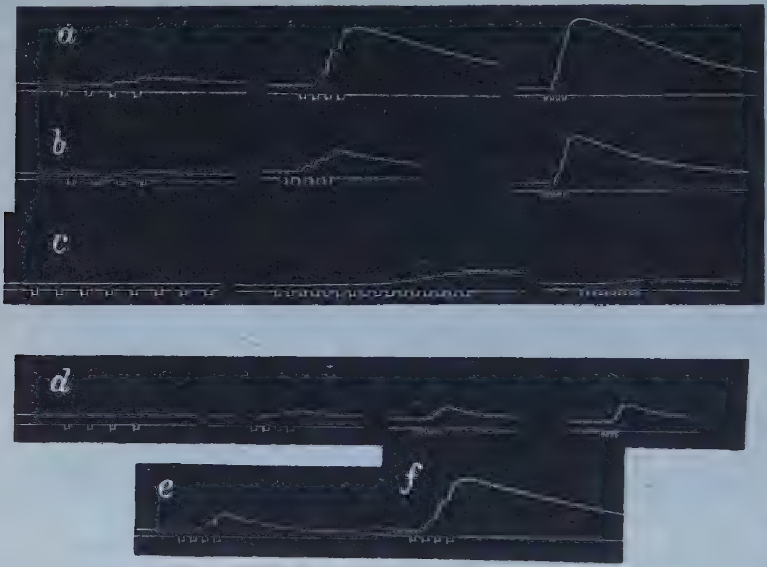

Fig. 2. (*a*) Responses from sphincter of *Calliactis* in sea water (stimuli 1 in 2 sec., 1 in 1 sec., 1 in 0·5 sec.). (*b*) The same after 28 min. in 50 % 0·4 *M* MgCl$_2$+50 % sea water. (*c*) The same after 55 min. (*d*) After 10 min. in sea water following 70 min. in the Mg solution (stimuli 1 in 2 sec., 1 in 1 sec., 1 in 0·5 sec., 1 in 0·35 sec.). (*e*) The same after 90 min. in sea water (stimuli 1 in 1 sec.). (*f*) The same after 24 hr. in sea water (stimuli 1 in 1 sec.).

Prolonged exposure to excess magnesium leads to breakdown of the rule that the response always begins on the second stimulus of a series. There is evidence that this is not due to modification of neuromuscular conduction but to the action of magnesium in breaking down synaptic junctions in the nerve net itself. The nerve net which serves the sphincter normally conducts without hindrance to all parts of this and of certain other muscles. But when exposure to magnesium has reduced the response to the condition shown in Fig. 2*c*, it is found that contractions even of the sphincter tend to be localized, particularly to that part of the animal nearest the electrodes. This means that barriers have been developed to free conduction within the nerve net itself. A study of a response such as that recorded to a frequency of one shock per second in Fig. 2*c* bears this out. No response took place till the third stimulus. Some minute responses then occurred up to the tenth stimulus. But they only occurred to every alternate stimulus, as though the conduction path to the muscle periodically failed. After the tenth stimulus, however, a path of conduction to the muscle became established and each stimulus produced a response.

Recovery from the breakdown of the through conduction of the nerve net itself is much more rapid than recovery from the depression of neuromuscular facilitation. Fig. 2*d* shows that even 10 min. in sea water after over 1 hr. exposure to excess magnesium suffices to restore normal through-conduction in the nerve net, while neuromuscular facilitation is still greatly depressed. Complete recovery from magnesium depression takes over 12 hr.

It is interesting to note in parenthesis that the "after-discharges" or adventitious responses which at times occur in normal animals during a series of stimuli, still make their appearance both under carbon dioxide and under excess magnesium. This precludes a possibility that they are due to impulses casually arising from superficial sense organs (Pantin, 1935*b*), because in Mg at least these are anaesthetized. It also reduces the likelihood that they are due to enhanced excitability of parts of the nerve net, because both these substances diminish excitability. These facts lend colour to the idea that "after-discharges" may be due to nervous impulses which originate from the same stimulus, reaching the muscle by two independent paths in the nerve net of very different conduction rate.

POTASSIUM

The effects of excess potassium on the responses of *Calliactis* are profound and somewhat unusual. They are completely reversible even after several hours' exposure of the animal to 50 % 0·6 *M* KCl with sea water. Immersion in sea water containing 5 % of added isotonic KCl (0·6 *M*) immediately causes spasmodic contraction of the tentacles and repeated contractions of the sphincter. These soon become less frequent though for some time there is a tendency for adventitious nervous impulses to appear. But the most striking effect is a huge augmentation of the size of the contractions which rapidly develops during the first 30 min. of immersion (Fig. 3). Recovery is complete on return to natural sea water.

Despite the large size of the contractions there is little change in the character of the facilitation process. Each stimulus still produces a single contraction and the size of the contractions increases as the interval between stimuli is reduced. The only obvious difference is the greater size of the contractions at all frequencies compared with the normal. Occasionally, however, a distinct though minute contraction develops in response to a single stimulus.

Not only is facilitation still present, but the rate of decay of the facilitating effect of a stimulus is little altered from the normal. Fig. 3*a*, *b* shows that the response will vanish when the interval between a pair of stimuli is increased to about the same value both in a normal animal and when subjected to excess potassium. This is the more remarkable because under potassium even the smallest contractions are enhanced in size and are therefore more readily detected.

The large size of the contractions which take place under the influence of potassium is at least partly due to the prolonged maintenance of tension. Maximum tension is only reached several seconds after the stimulus. Indeed the contractions might be thought to be brief tetani through the stimulus setting up not one but several nervous impulses in the nerve net. This, however, cannot be true because if each stimulus gave rise to several impulses it would completely upset the norma facilitation phenomena; and there would also be a large response to the first stimulus. Moreover, each of the extra impulses would produce its own response in the muscles and these are not visible (Fig. 3).

The enhanced contractions may be due partly to increased excitability of the muscle fibres so that more are facilitated at each contraction. But their prolonge character is at least partly due to change of the mechanical response of the muscle

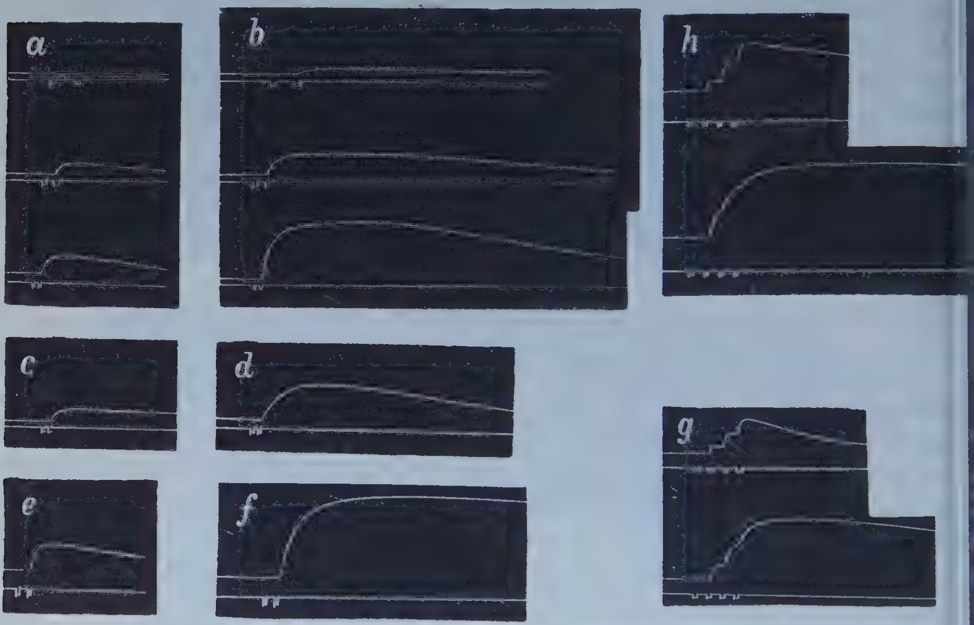

Fig. 3. (*a*) Response of *Calliactis* sphincter in sea water to pairs of shocks at 2, 1 and 0·5 sec. interval. (*b*) The same after 10 min. in 5% 0·6 *M* KCl + 95 % sea water. (*c*) Response of sphincter in sea water to pair of shocks at 0·5 sec. interval. (*d*) The same after 40 min. in Mg-free sea water. (*e*) Response of sphincter in sea water to pair of shocks at 0·5 sec. interval. (*f*) The same after 16 min. in sea water at *p*H 5·6. (*g*) Above: first response of sphincter in sea water to shocks at 1 per sec. Below: response 10 min. later, after animal was made to close five times by five volleys of ten shocks each, showing enhancement in early stages of fatigue. (*h*) Above: response of sphincter in sea water to shocks at 1 per sec. Below: the same after 35 min. in 5 % 0·6 *M* KCl + 95 % sea water. Note minute response to first stimulus.

The prolongation of the state of contraction resembles that of frog's muscle under veratrine. There does not seem to be an abnormally high intensity of contraction in the muscle fibres, for the maximum tension achieved after a series of stimuli when potassium is present is not far above normal, though fewer stimuli are required to reach this maximum.

This "veratrine-like" effect of excess potassium in actinians appears to be unique. In the muscles of many animals excess potassium gives rise to prolonged contracture, especially in unstriated muscle (Wells, 1928; Gasser, 1930). But in these cases there is a direct contraction in response to the solution, not one which waits upon a stimulus for its appearance. Nevertheless, this may only be a difference of degree for very high concentrations of potassium (10–50 % 0·6 M KCl in sea water) do cause direct contraction of the whole musculature.

POTASSIUM AND MAGNESIUM ANTAGONISM

In the skeletal muscle of *Carcinus* Katz (1936) showed that excitation by excess potassium is prevented by magnesium. In *Calliactis* the actions of both these ions are intimately connected. Magnesium-free sea water influences the response of the sphincter in the same way as sea water containing excess potassium (Fig. 3*c*, *d*). Potassium-free sea water resembles in its effects sea water to which magnesium has been added. The effect of any solution depends on the balance between these two cations. Excess magnesium diminishes the enhanced responses due to excess potassium. In high concentrations (50 % 0·4 M $MgCl_2$ with sea water) it will prevent the spontaneous contraction caused by high potassium concentrations (12 % 0·6 M KCl in sea water, and above).

We have already shown that magnesium acts at the neuromuscular junction. We have also shown that enhanced responses under excess potassium are not due to excitation of the nerve net. Since magnesium can diminish or prevent the action of potassium it appears that this also must exert its effect at neuromuscular junction.

THE HYDROGEN ION

Though a moderate increase in acidity (pH 6·5) produces little effect on the response of *Calliactis*, a point is reached in the neighbourhood of pH 5 at which the contractions of the sphincter in response to stimulation are greatly enhanced in size and duration. The whole action is parallel to that of excess potassium and the process of facilitation is unaltered except for the size of the contractions (Fig. 3*e*, *f*).

FATIGUE

If a resting *Calliactis* is caused to respond to several batteries of stimuli in succession the response shows fatigue. The contractions become longer and slower and their size may for a time become considerably increased. Repeated excitation of a muscle must lead to some accumulation of hydrogen ions and of carbon dioxide. Excited tissues also tend to lose potassium to the surrounding tissues (Dulière & Horton, 1929). Such an accumulation of carbon dioxide, hydrogen ions and potassium ions would lead to changes in the response like those actually observed in the early stages of fatigue.

CALCIUM

Increase in the calcium content of sea water causes large augmentation of the contractions of the sphincter (Fig. 4). The calcium contractions are quite normal in appearance except for their size. There is no prolonged contraction like that caused by the potassium and hydrogen ions. The effects of even high calcium concentrations are completely reversible in normal sea water.

Fig. 4 shows that the facilitation phenomena are normal. The response increases steadily as the stimulation interval is diminished. With excess calcium there is no evidence of even a slight response to a single stimulus. The interval which jus suffices to permit the facilitating effect of a stimulus to die completely away, so tha a following stimulus is ineffective, is about the same as in normal animal. Calciun does not influence the rate of decay of facilitation.

Calcium seems to exert its effect at the neuromuscular junction. As in the cas of potassium, its augmenting effect is greatly depressed by excess magnesium Since magnesium exerts its effect on the neuromuscular junction, calcium mus therefore act either at this point or at some earlier point in the chain of event between stimulation of the nerve net and the contraction of the muscle. But just a in potassium, the character of the responses, particularly the maintenance o facilitation, makes it clear that augmentation by excess calcium is not caused b extra nervous impulses set up in the nerve net. The only other place at which th calcium can be acting is between the nerve net and the muscle.

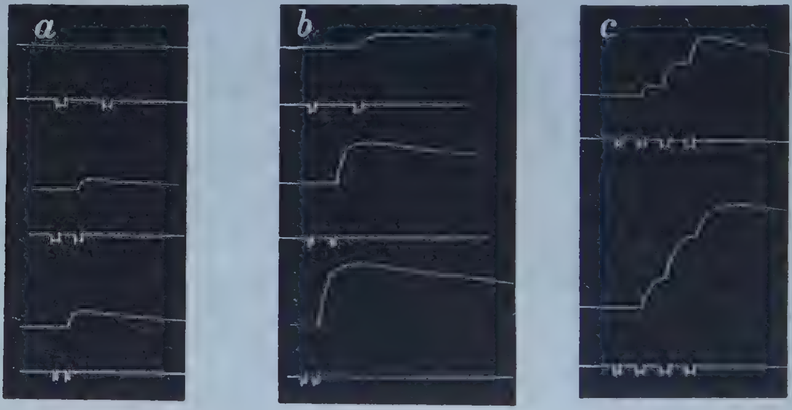

Fig. 4. (*a*) Responses of *Calliactis* sphincter in sea water to stimuli at 2, 1 and 0·5 sec. interval (*b*) The same after 60 min. in 5% 0·4 *M* $CaCl_2$+95% sea water. (*c*) Above: response of sphincter in sea water to stimuli at 1 per sec. Below: the same after 30 min. in 5% 0·4 *M* $CaCl_2$+95% sea water.

We have mentioned that facilitated responses of the muscles of anemones bea an analogy to those of vertebrate skeletal muscle during partial curarization. This analogy is more than superficial. Excess calcium enhances the facilitated response while excess magnesium depresses it; that is, calcium decreases the "curarization". The curarizing action of magnesium on vertebrate muscle is well known, and Feng (1936*a*, *b*) has shown how calcium antagonizes the action of a very large number of curarizing substances, including magnesium. The parallel with augmentation of facilitation by calcium in *Calliactis* is clear.

DISCUSSION

The influences of the substances described in this paper are confined almost entirely to the facilitated response. High concentrations of potassium alone directly excite contraction of the muscles. The calcium and hydrogen ions, and the potassium ion in lower concentrations, only augment the response to stimuli, while carbon dioxide and magnesium only depress it. These substances appear to act at the neuromuscular junction, and their mode of action is curiously limited. The rule that a single stimulus fails to produce a response holds in all cases except for the trivial contractions which sometimes occur under the influence of potassium. And in spite of great modification of the size of the contractions no ion seriously changes the rate at which facilitation decays. This contrasts with the immense effect of temperature on the decay.

These facts throw light on the method by which facilitation takes place in sea anemones. There are at present two general views concerning transmission at neuromuscular and other junctions in different animals. According to one, excitation is directly transmitted by the action current. According to the other, the nervous impulse liberates some transmitter substance, which in turn excites the muscle fibre. Monnier (1936) has suggested that at least in some muscles both these methods of excitation may co-exist. But whichever of these views is taken it would appear that excitation of the muscle is brought about by one factor, whether it is the nervous impulse or a chemical transmitter. It may be, however, that the process of transmission sometimes involves more than one factor. In summarising their conclusion on transmission in vertebrate systems Dale, Feldberg & Vogt (1936) suggested two alternative hypotheses: (1) that a transmitter substance directly excites the muscle cell, and (2) that the nervous impulse excites the muscle cell but cannot reach it unless the latter is sensitized by a chemical substance produced at the nerve ending. It is not intended to discuss the validity of these hypotheses for vertebrate systems, but they illustrate two possible methods of neuromuscular action. The first hypothesis involves only one factor in excitation, the second involves two; a process of sensitization and a process of excitation which it must precede. Clearly there are many conceivable hypotheses of this class in addition to the particular one mentioned above: either or both processes may be either electrical or chemical. Let us consider to which class the facilitated transmission of actinians belongs.

At first sight the step-like rise in tension caused by a series of shocks strongly suggests a response to successive increments of a transmitter. The effect is in a great measure due to simple mechanical summation. Each stimulus in a series adds to the tension in the viscous muscle while relaxation after each increment is slow. Nevertheless a series of stimuli does progressively augment facilitation. Fig. 5 shows the size of the response of the sphincter of *Calliactis* to the second stimulus of a pair separated by increasing intervals of time. The facilitating effect of the first stimulus (itself ineffective) and its decay are seen. If, however, a rapid series of stimuli is given instead of the single first stimulus of such a pair, facilitation can be piled up to such an extent that it takes a far longer time to decay and causes a much bigger response, except where the facilitated contractions are maximal. The second curve in Fig. 5 shows the response to a stimulus that has been preceded not by a single stimulus as in the first curve but by two stimuli at 0·5 sec. interval. It shows the larger size of the responses and the prolonged decay. If there are more than two preceding stimuli the effect is still greater. Facilitation is therefore added to by each stimulus of a series and dies away between each. How far is it consistent with the simple transmitter theory?

It is remarkable that a single stimulus applied to an anemone is normally without effect upon the muscles. On the basis of a simple transmitter hypothesis, this failure to respond to a single stimulus must mean that the exciting substance is produced in less than threshold quantity. The response to a second stimulus given soon after the first must then be due to the addition of further exciting substance so that the threshold is exceeded. In such a system factors such as excess calcium which enhance facilitation might enable a clear response to take place at the first stimulus, while factors such as excess magnesium which sufficiently depress facilitation must make it necessary for several stimuli to be given before sufficient transmitter accumulates to cause a response. Yet apart from the occasional trivial responses under potassium, neither ionic changes nor many other changes alter the rule that a single stimulus produces no response, and the response begins on the second stimulus if it takes place at all, so long as the pathways in the nerve net remain intact.

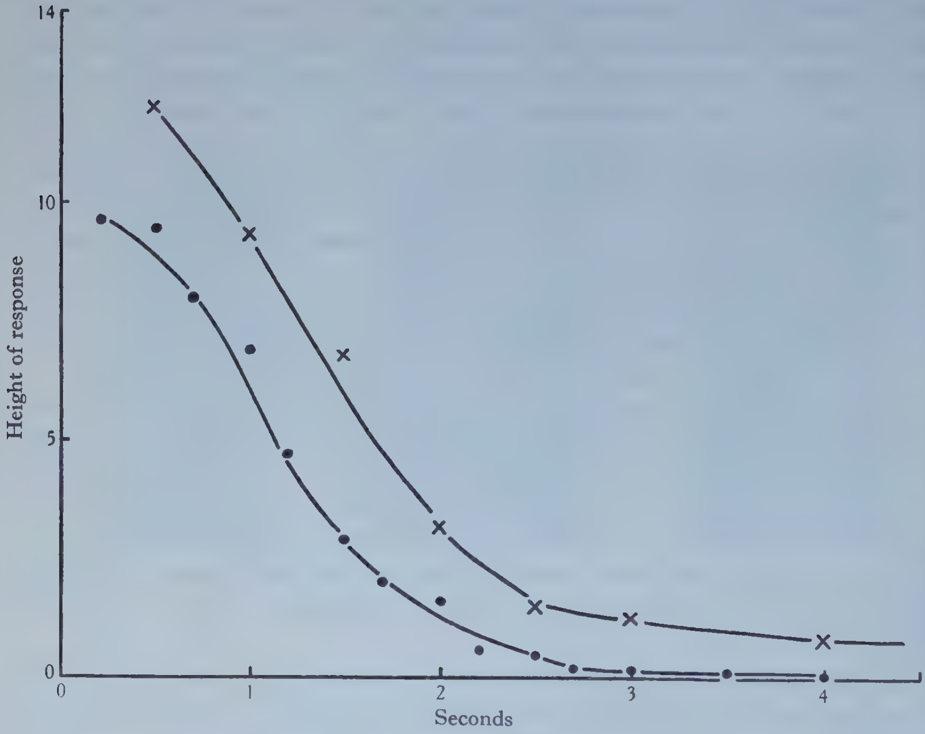

Fig. 5. Abscissa: interval between a pair of stimuli. ● Response to second stimulus of a pair. × The same response when the first stimulus of the pair is preceded 0·5 sec. by a preliminary stimulus.

These facts suggest that facilitation in anemones is not brought about simply by a transmitter. This can be tested in the following way. Suppose a single stimulus produces a transmitter in less than threshold amount. Each subsequent stimulus of a series will add to it. But since facilitation decays after each stimulus the rate at which transmitter piles up towards the threshold will depend on the frequency of stimulation. When this is high the threshold will be exceeded at the second stimulus. But if the interval between stimuli is lengthened there will come a point at which the amount of transmitter after the second stimulus just fails to reach the threshold, while a third stimulus at the same interval will enable this to be passed. Consequently the response will only begin at the third stimulus of the series.

Table I gives the size of the contractions of the mesenteric retractor of a *Metridium senile* in sea water to a series of three stimuli at different frequencies. The size of the contraction is measured in arbitrary units (mm. of the smoked record). It represents the added tension following each shock, not the total tension at the moment. The response was recorded with a weak isometric lever.

Table I. *Temperature at* 20·0° C.

Interval between shocks sec.	Height of contraction		
	To 1st shock	To 2nd shock	To 3rd shock
0·30	0	16·0	12·0
0·40	0	22·7	18·0
0·80	0	13·6	14·6
1·20	0	7·4	11·8
1·60	0	1·8	3·8
1·80	0	0·8	1·6
2·00	0	0·5	0·9
2·20	0	0·1	0·2
2·60	0	<0·1	<0·1
2·80	0	Trace	Trace
3·20	0	0	0

It will be seen that as the interval between stimuli is lengthened, the response to the third stimulus disappears at the same time as the response to the second. It is not possible to obtain two apparently ineffective stimuli before the first response as the simple transmitter hypothesis would lead one to suppose. On the other hand, these results can be easily explained if it is supposed, in accordance with the second hypothesis, that the first stimulus is ineffective because it finds the neuromuscular junctions unsensitized.

Fig. 6 illustrates the same question in a different way. It shows the size of response of the sphincter of *Calliactis* to pairs of stimuli of increasing intervals. The response to the second stimulus disappears in this experiment at a time interval of about 3·0 sec. On a simple transmitter hypothesis there should still be sufficient transmitter left after 3·0 sec. to bring the total amount with that added by a second stimulus almost to the threshold value. If therefore a pair of stimuli is preceded by an extra stimulus 3·0 sec. earlier, the response obtained should be substantially higher than without this preceding stimulus; for in this case the leading stimulus of the pair brings the amount of transmitter just to threshold value, while without any preceding stimulus it is well below it. To be on the safe side, the preceding stimulus was sent in 2·4 sec. before the pair, so that its facilitating effect had not quite vanished. In spite of this the presence of the preceding stimulus makes no obvious difference to the size of response. This is incompatible with the simple transmitter hypothesis.

A further difficulty for this hypothesis depends on the fact that in anemones the contraction is built up by responses of the muscle directly corresponding to each stimulus received. In considering facilitation in *Carcinus* muscle, Katz (1936) pointed out that accumulation of a transmitter must soon lead to numerous extra responses of the muscle or indeed to continuous activity. Yet in anemones even maximal responses are built up of contractions corresponding to the arrival of each nervous impulse at the muscle. This remains true even when the facilitation is enhanced by calcium or potassium.

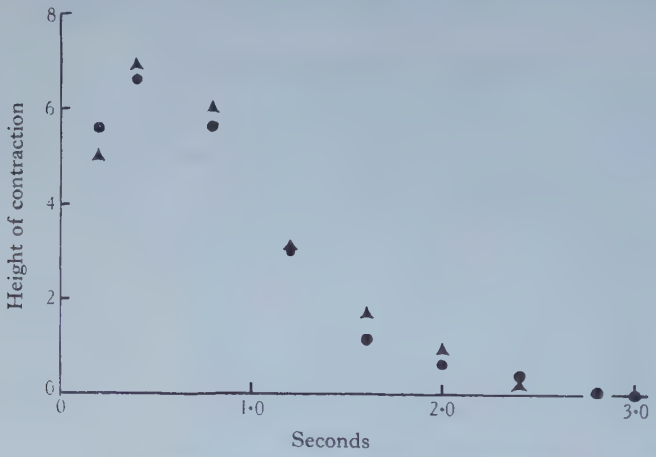

Fig. 6. Ordinates: height of response. Abscissa: interval between stimuli. ● Response to second stimulus of a pair. Last trace of response at 3·0 sec. ▲ Response to similar pair of stimuli preceded by a single stimulus 2·4 sec. earlier.

All these facts are easily explained if two distinct processes are involved in neuromuscular transmission. In this case a single stimulus fails not because a transmitter is present below threshold concentration, but because the first stimulus does not find the neuromuscular junction prepared for transmission. The stimulus nevertheless leaves behind it a state of sensitization which enables a subsequent stimulus to be effective. Further, each stimulus of a series can increase the state of sensitization of the junction, thus making larger responses possible. But the muscle will only contract in response to each excitation process.

The nature of the excitation and sensitization processes governing transmission in these actinian muscles remains to be determined. The influence of ions like potassium and magnesium on the response is not inconsistent with excitation being due to the action current itself, for these ions affect the conduction of excitation (Cowan, 1934; Katz, 1936). On the other hand the high temperature coefficient of the decay of the facilitation, the lack of influence upon it of ions which affect the electrical conditions at the cell surface, and the fact that facilitation may endure in some cases for more than 100 times the relative refractory period of the nerve net, suggest that the sensitization process may be due to the accumulation of an actual substance rather than an electrical state of the kind found by Bremer (1931) under certain conditions in vertebrate muscle.

In *Carcinus*, Katz suggested that potassium might take part in neuromuscular transmission. In anemones potassium is certainly not the facilitating substance itself. For in the presence of an excess of that substance a single stimulus must suffice to cause strong contraction of the muscle. But the possibility that a "facilitator" truly exists, and that it is chemically comparable to the "transmitters" found in the tissues of other animals will be discussed by one of us in a subsequent paper.

SUMMARY

1. The anaesthetic effects of carbon dioxide and magnesium added to sea water on *Calliactis parasitica* and *Metridium senile* have been studied. Magnesium first paralyses sense organs, but the main effect of both magnesium and carbon dioxide is a depression of neuromuscular facilitation. This prevents conduction of nervous impulses to the muscles in a way analogous to curarization of vertebrate skeletal muscle.

2. The chief effects of excess calcium, potassium and the hydrogen ion are increases in the size of the facilitated response to stimulation. The responses under potassium and the hydrogen ion are greatly prolonged and resemble the veratrine contracture of vertebrate skeletal muscle.

3. All the substances studied exert their chief effects at the neuromuscular junction. Analysis of their mode of action indicates that neuromuscular transmission in anemones involves two distinct processes, a process of excitation and a process of sensitization of the neuromuscular junction without which excitation of the muscle by the nervous impulse cannot be effective. This view is confirmed by examination of the relation of the size of the responses in normal animals to successive stimuli. The nature of the sensitization process and of the excitation process is discussed.

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ON THE EXISTENCE OF TWO TYPES OF CHROMATIC BEHAVIOUR IN TELEOSTEAN FISHES

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(With Seventeen Text-figures)

I. INTRODUCTION

It is well known that colour changes of animals may be brought about by (*a*) direct action of light, heat and other stimuli on the skin chromatophores, as, for example, in the "primary" photic response of Amphibia, and (*b*) action of stimuli on localized receptors.

Co-ordinated responses, i.e. responses of the second type, fall into two categories which offer a striking initial contrast in their time relations.

(i) *Rapid* type, e.g. the responses of the chameleon to local electrical stimulation and of the minnow to light reflected from its surroundings.

(ii) *Slow* type, e.g. the photic responses of Crustacea, Amphibia, Cyclostomes and Elasmobranch fishes.

II. SIGNIFICANCE OF TIME

In any co-ordinated response, a cycle of behaviour involves (*a*) the time taken for the stimulus to act on the receptor, (*b*) the time taken for the distribution of a disturbance through the co-ordinating mechanism, (*c*) the time taken for the effector to execute its response.

Where the eye is the receptor (*a*) is at most a matter of seconds.

If the mechanism is a nervous one (*b*) involves (i) transmission of the nervous impulse along the nerve trunks concerned, (ii) delay at synapses, (iii) delay at the nerve effector junction. A maximum distribution time value for these taken together is also at most a matter of seconds.

The maximum time for (*a*) and (*b*) together may therefore be taken as under 1 min., and any excess within the period of the complete cycle must be due to (*c*).

If, therefore, it is found that the total time for complete colour change, i.e. for the complete cycle, is of the same order as the effector-reaction time (*c*), then there is nothing to suggest that the co-ordinating mechanism is other than the nervous reflex.

If, however, the total time for the colour change is markedly greater than the effector time, then some other co-ordinating mechanism must be involved, e.g. the reflex liberation of hormone and its gradual accumulation in the blood stream to a certain limiting value.

Effector-reaction time can be assigned a maximum value on the basis of experiments with substances which bring about "contraction" or "expansion" of melanophores. For example, Osterhage (1932), employing a wide range of tissue extracts and other substances, found that the time taken to bring about contraction or expansion of melanophores from an intermediate state in *Gasterosteus* and *Rhodeus* ranged from 6 to 50 min. More recently Waring (unpublished), from perfusion experiments with fresh pituitary posterior lobe extract, has found the reaction time of fully contracted dermal melanophores in *Xenopus laevis* to be 75 min. for complete expansion. Vertebrate melanophores in general appear to react rather slowly as compared with muscle.

It may be concluded, therefore, that where the total time taken for colour change exceeds two hours hormonal co-ordination is indicated. On the other hand, a total change time of the order of 10 min. or less may be safely held consistent with predominantly nervous co-ordination, through the direct innervation of the melanophores themselves.

Within these limits, at least, the time relations of the chromatic behaviour of any vertebrate animal furnish a guide to the type of co-ordination involved.

The behaviour of dermal melanophores only is referred to in the present paper. Melanophores were assessed on the scale first introduced by Hogben for Amphibia. On this scale a melanophore index (μ) of 1·0 denotes complete contraction (aggregation of pigment) and a melanophore index $\mu = 5{\cdot}0$ denotes complete expansion (dispersion of pigment).

III. MELANOPHORE READINGS

As has been stated, the pigmentary effectors dealt with in this paper are the large dermal melanophores, analysis of whose mechanism is sought. Each recorded point of the time graphs presented is the mean of a number of animals whose melanophore indices were assessed on the above-mentioned scale after careful microscopic examinations of the state of a number of individual *dermal* melanophores, at a constant site, in the *living animal.*

This allows the state of the dermal melanophores at any given moment to be recorded quantitatively and accurately, irrespective of the general colour or shade of the whole animal.

In the eel, for example, two other classes of pigmentary organs at least are present in the skin—epidermal melanophores and xanthophores. All three classes of pigmentary effectors contribute to the naked eye or visible appearance of the animal.

The following protocol is relevant to this point. No two of the animals presented quite the same appearance to the eye.

Eel, white tank, 9° C. 168 *hr.*

Dermal melanophores	Epidermal melanophores	Xanthophores	Naked-eye appearance
2·5	1·5	1·5	Very pale
3·0	2·0	1·5	Medium
3·0	2·0	1·0	Pale
3·5	3·5	1·0	Dark medium
3·5	3·5	2·0	Darkish
3·5	3·0	2·5	Quite dark

The three classes of effectors must therefore be examined separately when analysis of their mechanism is undertaken, otherwise serious error is bound to occur. Hogben's criticism of the photo-electric method of A. V. Hill and his co-workers (Hogben, 1936) is clearly justified. Hill's method has been tried and subsequently discarded by Wykes (1937) for small teleosts. Wykes aptly observes, "photo-electric measurements of this type record the *sum effect* of colour response only and give us no information as to the activity of the different pigmentary effectors concerned".

Wykes's own quantitative method—the measurement of *fixed* melanophores—is also, however, open to criticism. It involves killing the small fish used by immersion in boiling water or in formalin. In the present state of knowledge of the cytological behaviour and effector-reaction time of vertebrate melanophores this seems risky, especially where the existence of nervous control may be known or suspected. Further, in fixing generally a certain degree of change of size is involved, which may vary with the state of the tissue concerned. Melanophore readings will therefore be slightly different from those made on the living animal; and if a curve has a very protracted asymptote—for example, those of the eel in the regions between 4·0–5·0 and 2·0–1·0, it is far from certain that this will appear accurately in a graph based on fixed material.

In Amphibia at least the fixed condition of fully expanded melanophores is invariably *less than fully expanded*; and the fixed condition of fully contracted melanophores is always *less than fully contracted*. A melanophore index of 5·0 becomes 4·0 and a melanophore index of 1·0 becomes 2·0 (Hogben, unpublished). The risk of error in this case may be judged from Fig. 1, which represents a hypothetical case where readings from living material give the curve *A*. The dotted line (*A'*) shows the curve resulting from readings of the same material *after* fixation.

Where chromatic behaviour is of the slow type and the husbandry of the animals concerned is well understood, examination of the melanophores of the living animal at intervals may be safely undertaken.

Where, however, chromatic behaviour is of the rapid type accurate observation of the melanophores of the living animal frequently presents technical difficulties. The sensitiveness of some animals, e.g. many teleostean fishes, to chemical and mechanical stimuli seriously increases the difficulty of recording precisely the state of the melanophores at a given moment, and will readily bring about abnormal chromatic behaviour, unless precautions are taken.

A satisfactory method in such cases is one where the melanophore changes of single animals *in situ* are followed through to equilibrium under a bar binocular microscope without disturbance of the animal in any way. The mean melanophore indices at given time intervals of a number of animals thus read under uniform conditions furnish points of a representative curve.

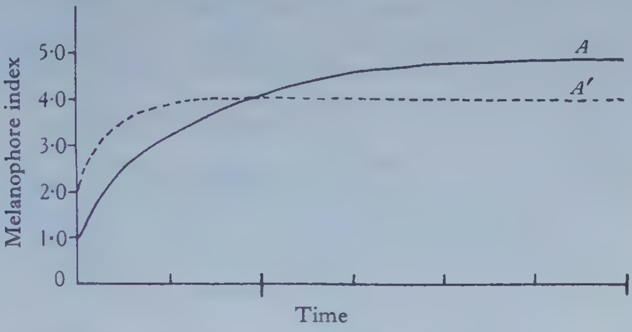

Fig. 1. Diagrammatic figure showing the curve resulting from melanophore readings on living (A) and fixed (A') material.

Alternatively, or where the foregoing method is not feasible, or is impossible, e.g. in determining chromatic behaviour in total darkness, the method of Hogben and Landgrebe (in press) for observations on the chromatic behaviour of *Gasterosteus* may be adopted. By this method each point on the curve represents the mean melanophore index—read in the most suitably speedy fashion—of a number of living animals, e.g. six, used *once*, i.e. used for that point only and then discarded.

All three methods of determining the melanophore changes of the living animal have been used in the present research and are referred to in appropriate context.

IV. THE CHROMATIC BEHAVIOUR OF THE EEL (*ANGUILLA VULGARIS* L.)

Of the two contrasting types of behaviour known amongst Vertebrates—already referred to as *rapid* and *slow* respectively—rapid change is exhibited by some teleostean fishes and some reptiles and there is in these groups direct evidence of direct nervous control of melanophores: slow change, for which there is direct evidence of pituitary control, occurs in cyclostomes, elasmobranch fishes and Amphibia.

On the basis of existing work—especially that of Young—the latter is the archaic form of the chromatic function.

Petersen, whose work on the zoology of the eel is well known, wrote in 1894 of "eels, like most other fishes, being able to change colour quickly". Examination of the time relations of colour change in the eel shows that this is not so, and indicates that the eel, though a teleost, is, as regards its chromatic behaviour, of the physiologically archaic type.

(1) *Procedure*

The time graphs shown in Figs. 3–7 result from experiments with eels 45–70 cm in length done under standardized conditions, using a constant artificial illumination i.e. a 25 W. bulb 30 in. above the bottom of each tank, the top of the tank being covered with a sheet of ground glass. All other light was excluded. Animals were kept in porcelain tanks in running water throughout. The slowness of the colou response of the eel allows successive readings at intervals of the melanophore

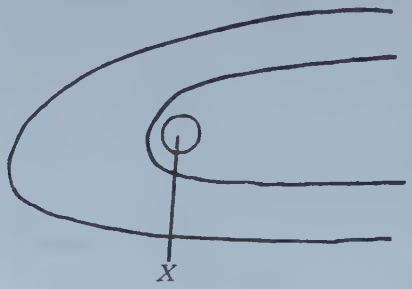

Fig. 2. Eel tail. X = site of readings.

indices of a group of animals to be undertaken under the conditions referred to without undue risk of error, and no difficulty was experienced in maintaining the animals over prolonged periods so long as they were properly handled. The following technique was adopted for reasons stated.

An eel was lifted from the tank by a capacious close-meshed net made of *soft* string, thick enough to remain sodden and flaccid. This was laid down on a solid surface, e.g. the glass top of an adjacent tank, and a *damp* cloth immediately laid over the animal, especial care being taken that the head was well covered. Thus treated animals readily become quiescent, and cloth and eel may be picked up and bent into a U shape convenient for holding, the tail-tip only protruding from the mass. This can only be done, however, without considerable resistance from the animal, provided that the head is completely but loosely covered, the action rapidly performed, and only *very light pressure* is employed. A firm grip invariably provokes resistance, and if persisted in will very frequently result in the death of specimens within a few days. The use of *dry* cloths invariably results in considerable mortality, owing to damage to the external mucus layer.

In the case of the larger specimens used in the present investigation, the mass of eel and cloth, too large for holding conveniently in one hand, was held lightly under the left arm, resting on the forearm and hand, while the tail-tip was read.

Reading was done by placing the tail-tip on wet glass plate covering the stage of a binocular microscope with concealed substage lamp; and thereafter the animal was released at once into its tank.

All readings were made at the site X (Fig. 2).

(2) *Chromatic behaviour*

The relevant time intervals are those involved in changing:

(*a*) From equilibrium on white background to equilibrium on black background in light.

(*b*) From equilibrium on black background to equilibrium on white background in light.

(*c*) From equilibrium in total darkness to equilibrium on white background in light.

(*d*) From equilibrium in total darkness to equilibrium on black background in light.

(*e*) From equilibrium on white background to equilibrium in total darkness.

(*f*) From equilibrium on black background to equilibrium in total darkness.

The mean equilibrium values of the eel were found to be:

	Melanophore index
On white background in light	1·2
On black background in light	4·8
In total darkness	3·5

Blinded eels kept for prolonged periods under standard illumination equilibrated at 3·7, thus showing a primary response of 0·2.

The response to change of background in light is very slow, taking 20 days on either white or black background at 8° C. (Fig. 3). A quite large rise of temperature has a relatively slight effect. Such effect is mainly seen in the early phases of equilibration This is shown in Fig. 4 where are reproduced the initial part of the time graphs of two groups of eels, one carried through at 22° C., the other at 7° C.

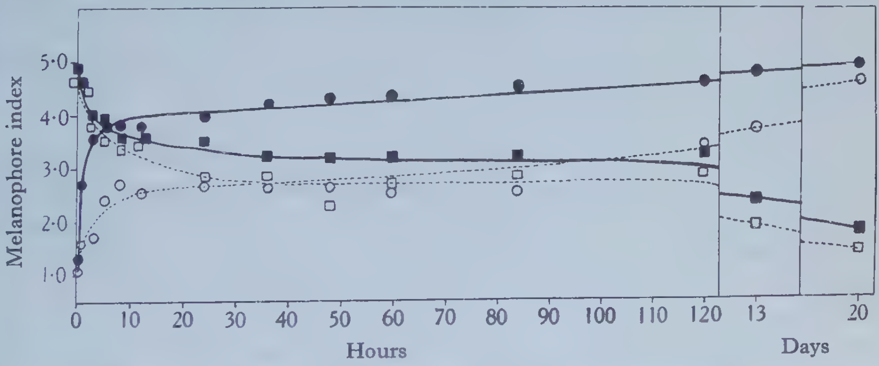

Fig. 3. Eel. Black and white background response in light. Temp. 8·3 ± 0·8° C. Standard lighting (see text). Each curve represents a group of six animals. Black circles and squares = dermal melanophores. Clear circles and squares = epidermal melanophores of the same animals.

As is well known in eels, the black and yellow pigmentation may give place to a black and silver coloration customarily believed to mark the onset of the reproductive phase of the life cycle. It would be more correct to state that this marks the onset of migration to the sea. In point of fact there is no difference between the state of the gonads of silver and yellow eels of the same size, and the statement that the silver and black coloration is the breeding dress is only true in so far as eels do not become sexually mature unless they do migrate.

The initial reactions of the dermal melanophores of the "silver" phase are in general very slightly faster than those of the "yellow" phase of the life cycle. These are shown in Fig. 5. The difference is almost wholly in the early stages of response. Apart from this the chromatic behaviour of the two phases is similar, so far as dermal melanophores are concerned.

A striking difference, however, in the silver phase as compared with the yellow occurs in a different class of pigmentary effectors—the epidermal melanophores. With the onset of the silver phase a considerable proportion of these exhibit irregular or patchy distribution of pigment within the melanophores. In the expanded melanophores the normal stellate appearance is destroyed by uneven dispersion of pigment. Under conditions inducing contraction aggregation of pigment may form a single mass or several. Further, in a proportion of the epidermal melanophores an appearance consistent with disruption of the melanophore supervenes and dispersal of its contents forms a grey blotch or blur of apparently inert pigment on and around the site of the melanophore.

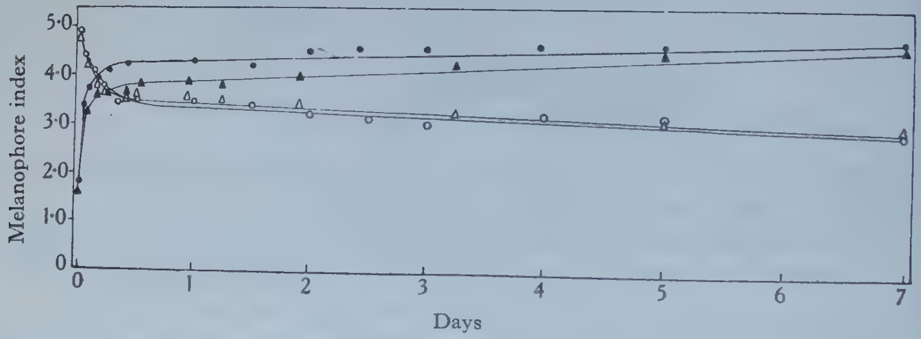

Fig. 4. Eel. Black and white background response in light at low and high temperatures. Each curve represents a group of six animals. Circles = at 6·7 ± 1·0° C., triangles = at 21·75 ± 0·5° C. Standard lighting.

The background responses in light of eels equilibrated for a prolonged period in total darkness and transferred to black and white background under standard illumination are shown in Fig. 6. Full pallor in white background is only attained after 22 days at 9° C. Complete darkening on black background takes 9 days at 6° C.

Eels transferred from a white or a black background in light to total darkness equilibrate at the intermediate figure of 3·5. Equilibration from white background is very slow, requiring 27 days at 8° C. Equilibrium is reached after 2 days from black background (Fig. 7).

The general similarity of these time graphs to those of Amphibia, e.g. *Xenopus laevis*, is very striking. The chromatic behaviour of both eel and *Xenopus* is of the slow type, though the latter is less slow in the final phases of equilibration.

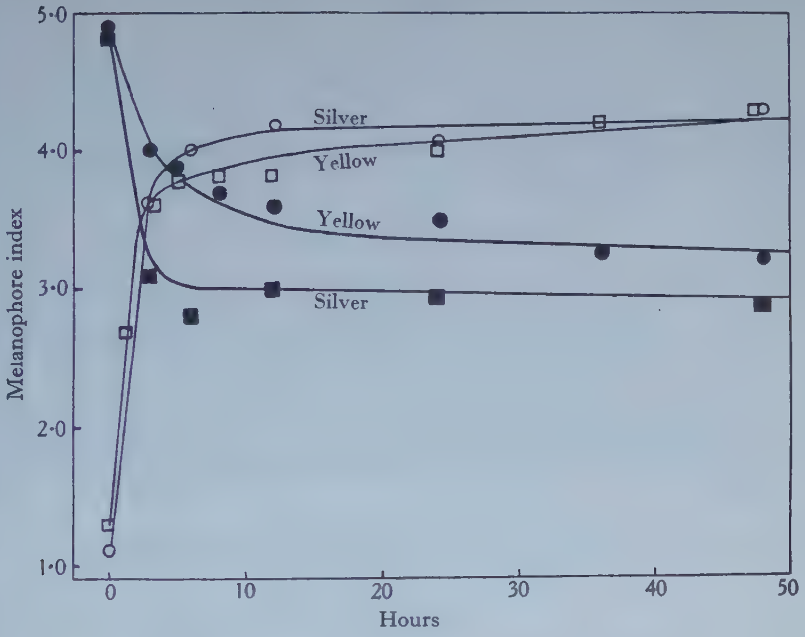

Fig. 5. Eel. Black and white background response in light of eels in the silver (squares) as compared with the yellow (circles) phase. Each curve represents a group of six animals. The early stages of the response are shown. The total time for complete response is similar in both phases. Temp.: silver 12·0 ± 0·4° C.; yellow 8·3 ± 0·8° C. Standard lighting.

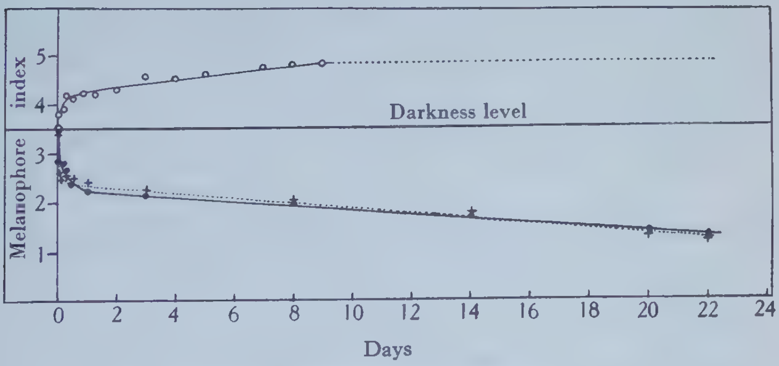

Fig. 6. Eel. Response to black (clear circles) and to white background (black circles) in light after 4 weeks in total darkness. Each curve represents a group of six animals. Temp. 6·0 and 9·0° C. respectively. Standard lighting. The dotted curve (crosses) represents a group of eels in the silver phase (temp. 9° C.).

The background responses of a group of *Xenopus* carried through unde identical conditions of lighting and temperature (6·7° C.) to one of the two group of eels recorded in Fig. 4 is shown in Fig. 8.

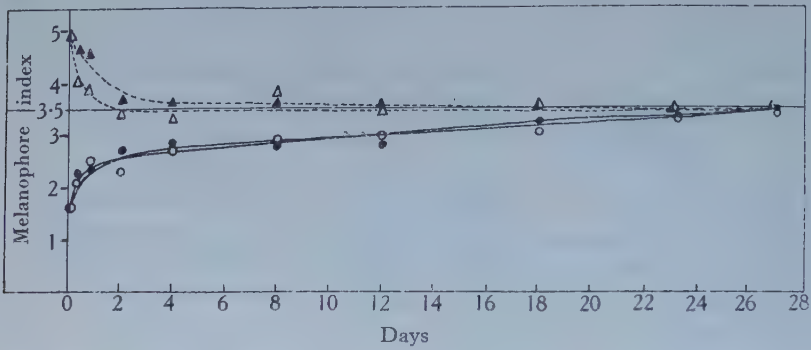

Fig. 7. Eel. Response on transference from black and from white background in light to tota darkness (clear triangles and circles). Each curve represents a group of six animals. Temp 8·5 ± 0·7° C. Black circles and triangles show similar animals blinded immediately prior to transference to darkness.

Both species exhibit similar reactions on change from black to white backgroun in light or vice versa. Comparison of Figs. 4 and 8 shows that in both there is a steep initial rise in reaction to black background and an absence of this steepness in reaction to white background.

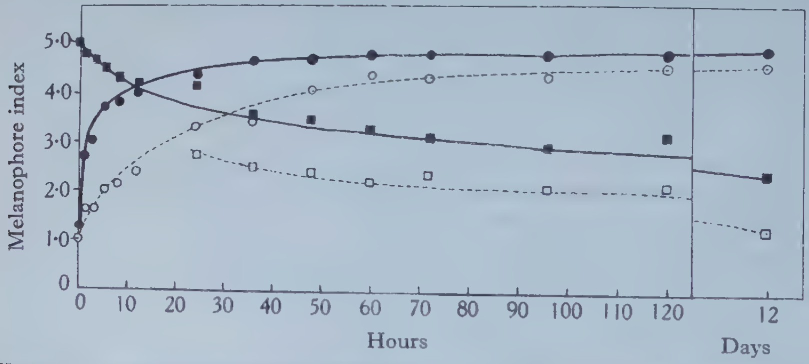

Fig. 8. *Xenopus*. Response to black and to white background in light of *Xenopus laevis* at 6·7 ± 1·0° C Standard lighting. Black squares and circles = dermal melanophores. Clear squares and circles = epidermal melanophores. Each curve represents a group of six animals.

The similarity of response is equally marked in animals equilibrated in total darkness and transferred to black and white background in light (eel, Fig. 6; *Xenopus*, in Fig. 4), Hogben & Slome (1931). In both species response to white background is slower than response to black background.

The similarity of behaviour is again borne out by the behaviour following transference to total darkness of eels and *Xenopus* equilibrated on white and black backgrounds in light (eel, Fig. 7; *Xenopus*, Fig. 4), in Hogben & Slome (1936). In both species the darkness equilibrium figure reached is intermediate, and in both the time taken to reach it from the white background condition is markedly in excess of that needed from the black background condition. In both also the primary response is small.

This correspondence is entirely consistent with what the time relations of the chromatic behaviour of the eel both in light and in darkness clearly indicate: that its chromatic behaviour is of the slow type where hormonal co-ordination is involved.

(3) *Nature of the mechanism*

It is evident that the chromatic behaviour of the eel is controlled by a hormonal mechanism, activated by localized photo-receptors in the retina.

In addition to bringing out which of the two alternative mechanisms previously discussed is concerned, the time relations of chromatic behaviour also throw light on the *complexity* of the mechanism involved, as shown by Hogben & Slome (1936) in Amphibia, Waring (1938) in elasmobranchs, and Smith (1938) in Crustacea.

(1) A convenient notation for the time intervals involved is as follows:

${}_bT_w=$ The time taken to change from equilibrium on black background in light to equilibrium on white background in light.

${}_bT_d=$ The time taken to change from equilibrium on black background in light to equilibrium in total darkness.

The relevant time intervals and the melanophore changes which accompany them are (using round figures for convenience):

Interval	Melanophore movement	Duration days	Interval	Melanophore movement	Duration days
${}_bT_w$	5·0 → 1·0	20	${}_wT_b$	1·0 → 5·0	20
${}_bT_d$	5·0 → 3·5	2	${}_wT_d$	1·0 → 3·5	27
${}_dT_w$	3·5 → 1·0	22	${}_dT_b$	3·5 → 5·0	9

In chromatic behaviour of the slow type either a single hormone is involved or more than one.

(*a*) If a single hormone is involved two alternative hypotheses present themselves:

(i) That the behaviour is controlled by a *melanophore expanding* hormone B, whose production is inhibited on a white background and augmented on a black background. This implies that the hormone concentration is highest on a black background ($\mu=5{\cdot}0$), least on a white background ($\mu=1{\cdot}0$) and intermediate in darkness ($\mu=3{\cdot}5$).

Since ${}_wT_b$ is the time taken to raise μ from 1·0 to 5·0, i.e. for maximum increase in concentration of hormone, neither ${}_wT_d$ nor ${}_dT_b$ can be greater than ${}_wT_b$, as they both involve smaller increases in concentration.

Since ${}_bT_w$ is the time taken to lower μ from 5·0 to 1·0, i.e. for maximum decrease in concentration of hormone, neither ${}_bT_d$ nor ${}_dT_w$ can be greater than ${}_bT_w$.

In the eel ${}_wT_d$ (27 days) *is* greater than ${}_wT_b$ (20 days), and ${}_dT_w$ (22 days) greater than ${}_bT_w$ (20 days).

Hypothesis (i) therefore fails.

(ii) That the behaviour is controlled by a *melanophore contracting* hormone *W* whose production is augmented on a white background and diminished on a blac background. This implies that the hormone concentration is highest on a whit background ($\mu=1{\cdot}0$), lowest on a black background ($\mu=5{\cdot}0$) and intermediate i darkness ($\mu=3{\cdot}5$).

Then since ${}_bT_w$ is the time taken to lower μ from 5·0 to 1·0, i.e. for maximu increase in concentration of hormone, neither ${}_bT_d$ nor ${}_dT_w$ can be greater than ${}_bT_w$ both involving smaller increases in concentration.

And since ${}_wT_b$ is the time taken to raise μ from 1·0 to 5·0, i.e. for maximu decrease in concentration of hormone, neither ${}_wT_d$ nor ${}_dT_b$ can be greater than ${}_wT_b$

The time curves of the eel show, however, that ${}_dT_w$ (22 days) *is* greater tha ${}_bT_w$, and ${}_wT_d$ (27 days) is greater than ${}_wT_b$.

Hypothesis (ii) therefore fails also.

The time relations therefore indicate that the hypothesis of a one-hormon mechanism will not cover the facts of colour change in the eel.

(*b*) If two hormones are involved the bi-humoral hypothesis put forward b Hogben & Slome (1931, 1936) to explain the chromatic responses of Amphibi may be next considered.

This hypothesis postulates that the melanophore index at any one time is du to the effective relative excess of one or other of two antagonistic hormones, eac with its own rate of production and elimination, one of which, *B*, is a melanophor expanding hormone and the other, *W*, a melanophore contracting hormone, eac activated by separate localized receptors in the retina.

If this hypothesis be well founded—as it has been shown to be in *Xenopu laevis*—the time relations of chromatic response are of primary significance.

The hypothesis implies that in an animal approaching equilibrium on a whit background in light production of both *B* and *W* will be going on, *W* over-riding *B*

The reactions taking place during the relevant time intervals may then b explained as follows:

		Initial state of melanophores	Final state of melanophores	Duration days
${}_wT_b$	Decrease of *W* (*B* increasing *in effect*)	1·0	5·0	20
${}_bT_w$	*B* constant. Increase of *W* (over-riding *B*)	5·0	1·0	20
${}_bT_d$	Decrease of *B*	5·0	3·5	2
${}_dT_b$	Increase of *B*	3·5	5·0	10
${}_dT_w$	Increase of *B*. Increase of *W* (over-riding *B*)	3·5	1·0	22
${}_wT_d$	Decrease of *B*. Decrease of *W*	1·0	3·5	27

Various degrees of interaction are therefore involved, implying that differen orders of time will be required for their completion.

(1) Since ${}_bT_d$ and ${}_dT_b$ each involve only a single process of production or elimination of hormone, expectation would be that these time intervals would be relatively short.

(2) Since ${}_dT_w$ and ${}_wT_d$ each involve two *competing* processes, which in the one case are, and in the other case may be, in active and direct competition with each other, expectation would therefore be that equilibrium would be reached much more slowly than in (1).

If the time relations of the chromatic behaviour of the eel are consistent with these implications of the bi-humoral hypothesis stated, then the order of duration of ${}_dT_w$ and ${}_wT_d$ should significantly exceed that of ${}_bT_d$ and ${}_dT_b$. Reference to the right-hand column of the foregoing table will show that this is so.

Examination of the time relations of the chromatic behaviour of the eel discloses therefore that co-ordination is effected by a humoral mechanism, and that while the hypothesis of a one-hormone control is inadequate to account for the time intervals observed, the latter are consistent with an hypothesis postulating two antagonistic hormones whose interaction in varying concentrations is responsible for the state of the melanophores existing at any given time.

(2) *Behaviour in darkness.* Complete expansion (dispersion) of the melanophores of the eel does not take place on transference to darkness from equilibrium on white or black background in light.

Where activation of humoral control of chromatic behaviour is by photic stimulation of retinal receptors, it might be expected that the degree of response would be dependent on the amount of light reaching the retina: and that therefore an animal transferred from a white background in light ($\mu = 1{\cdot}0$) to a black background in light (involving a reduction in the amount of light falling on the retina) would exhibit a less degree of response than one transferred from a white background in light to total darkness (*absence* of light). This is not the case. Instead, equilibrium in darkness occurs at $\mu = 3{\cdot}5$ and the time taken to reach it is *relatively protracted* in comparison with the time required in light for the same range of melanophore movement.

This suggests that melanophore expansion and contraction in darkness may be a physiologically distinct process from expansion and contraction in light. Presumably, therefore, the difference between the white background and black background responses in light is not due to light intensity, but to the action of light on different parts of the retina.

In any case we have to distinguish between two categories of visually determined photic response, i.e.

(*a*) the transition from equilibrium in light to equilibrium in darkness, and vice versa;

(*b*) the transition from equilibrium on a white background with overhead illumination to equilibrium on a black background with overhead illumination, and vice versa.

V. THE CHROMATIC BEHAVIOUR OF *LEBISTES RETICULATUS* (PETERS) REGAN

The existence of the slowly reacting archaic humoral type of chromatic mechanism in a teleostean fish, the eel, is of great interest, since the group is one whose reactions have been generally believed, since the work of Pouchet, to be under nervous rather than endocrine control.

Nervous control of melanophores has been demonstrated in a number o teleosts (Sand, 1935). Chromatic behaviour of the rapid type occurs amongs others in *Lebistes reticulatus* (Peters) Regan. Study of the time relations of *Lebiste* provides a contrast to those of the eel, as background response in light is complete in less than an hour.

(1) *Procedure*

The time graphs shown in Fig. 10 show the response of two series of six *Lebistes* (♀) equilibrated in small glass containers to black and white backgroun in porcelain tanks in running water, at 24 in. from a 40 W. opal bulb. On change of background the subsequent equilibration of each individual was followed through under a bar binocular without interference with the specimen. The time graphs shown in Fig. 11—equilibration to black and white background in light after 7 days' total darkness—were also constructed from readings made in this way. All readings of *Lebistes* were made at the site *X*, Fig. 9.

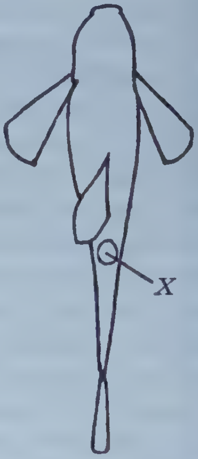

Fig. 9. *Lebistes*. *X* = site of readings.

In view of the rapidity of reaction of *Lebistes* to light, the curves shown in Fig. 12 were constructed differently. The fish were kept in total darkness in running water in small oval glass containers (Woolworth salt cellars) capped with a silk gauze cover secured by a rubber band. For reading, a container was gently lifted from the tank in darkness, a glass slip substituted for the gauze cap, and the container placed on the stage of a binocular microscope fitted with an enclose substage lamp, the beam of which was reduced by a diaphragm to a fine pencil On this lamp being switched on, rapid reading (5 sec.) of the melanophore index of the posterior region of the fish could be undertaken with a minimum risk o exposure of the specimen to direct light. At the same time a risk remained an accordingly each specimen was read *once* only and then discarded. Each point o the time graphs of Fig. 12 therefore represents the mean melanophore index o six individuals used for that occasion only. The starting figure of each batch o six was checked at the outset.

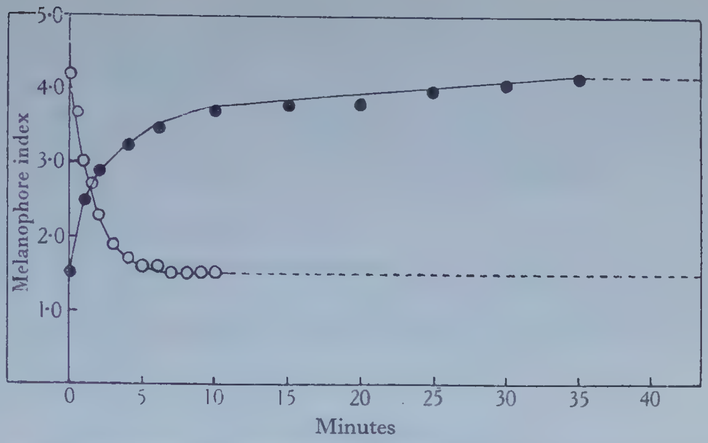

Fig. 10. *Lebistes.* Response to black and to white background in light. Each curve represents a group of six animals. Temp. 16° C. Lighting, 40 W. opal bulb at 24 in.

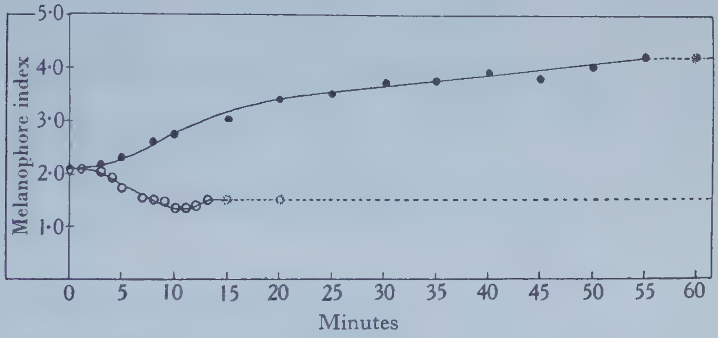

Fig. 11. *Lebistes.* Response to black and white background in light after 7 days in total darkness. Each curve represents a group of six animals. Temp. 16° C. Lighting, 40 W. opal bulb at 24 in.

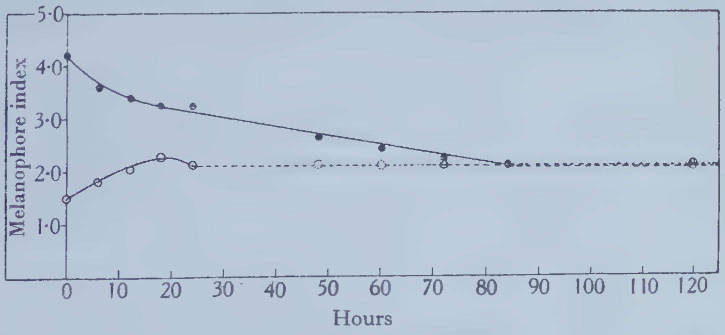

Fig. 12. *Lebistes.* Response on transference from black and white background in light to total darkness. Each point on the curves represents a separate group of six animals. Temp. 16° C.

(2) *Chromatic behaviour*

Mean equilibrium values	Melanophore index
On white background in light	1·5
On black background in light	4·2
In total darkness	2·1

Attempts to ascertain the primary response in *Lebistes* have been less conclusiv owing to the large mortality subsequent to removal of the eyes and before equilibriu may have been reached.

Eyeless animals kept under standard lighting behaved as follows:

		Surviving				
° C.	No.	48 hr.	72 hr.	96 hr.	7 days	10 days
16	20	5	4	4	2	1
Mean melanophore index		2·7	2·7	2·8	2·7	3·0

Since equilibration in total darkness takes place at 2·1 the range of the primar response would appear to be 0·6–0·9.

The response in light of *Lebistes* transferred from black to white backgroun and vice versa is rapid (Fig. 10), especially the white background response whic is complete in 7 min. Both black and white background responses slow down i the later stages of equilibration. In the former 35 min. in all are required fo equilibrium to be reached.

In *Lebistes*, transferred from total darkness ($\mu=2·1$) to black and to whi backgrounds in light, response is complete within 1 hr. (Fig. 11).

The behaviour in darkness of *Lebistes*, previously equilibrated on black and o white backgrounds in light (Fig. 12), shows a sharp contrast in *time* with its behaviou in light. Over 80 hr. are necessary for equilibrium to be reached following equilibration on black background, and 24 hr. from white background.

VI. THE CHROMATIC BEHAVIOUR OF *SALMO SALAR* L.

In the course of observations on the post hatching and subsequent parr stage of *Salmo salar* L and *S. trutta* L. it was noticed that the chromatic behaviour o these species appeared to be also of the rapid type. Observations on the latter als showed that background response does not occur in *Salmo* till approximatel 3 weeks after hatching, at 7–8° C. This is consistent with the fact that the development of the eye in *Salmo* remains incompleted for some time after emergenc from the egg.

For the time graphs presented in this paper *S. salar*, 3–4 months old (salmo parr), have been used.

(1) *Procedure*

The making of precise melanophore readings on these fish is rendered difficult by their extreme activity coupled with the rapidity under certain conditions of their colour response, and the readiness with which this may be disturbed by extraneous stimuli, e.g. by incautious manipulation. The following method was eventually used and found to work satisfactorily in practice.

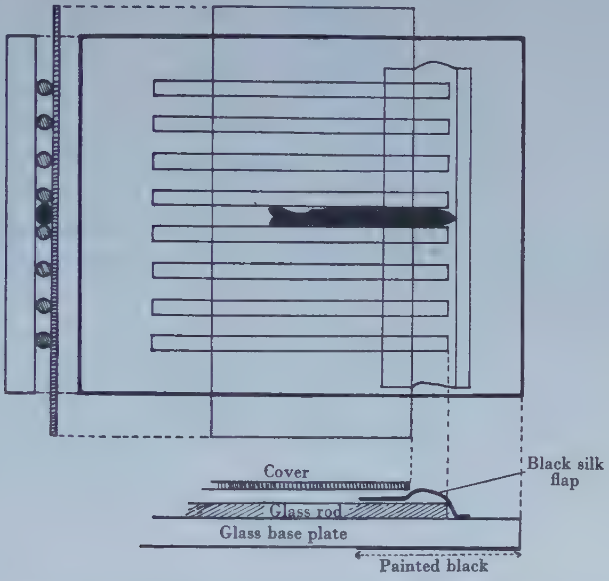

Fig. 13. Glass grid used for reading melanophores in living *Salmo*. Glass rods or lengths of tube are cemented to a glass base, the width of rods and interspaces being adjusted precisely to the dimensions of the animal. A flap of black silk gauze is cemented along one end, as shown. This acts as a stop when the fish is released on to the slightly tilted grid, surplus water draining away. A glass cover is placed over all, free of the anterior end of the fish. The underside of the base plate beneath the gauze flap is painted black.

Fish were equilibrated in running water in black and in white porcelain tanks in glass tubes capped with fine black or white silk gauze secured by a narrow rubber band, at 24 in. from a 40 W. electric bulb. For reading of the melanophore index a tube was taken from the tank, uncapped, and its contents poured rapidly, in subdued light, on to the glass grid shown in Fig. 13. A glass cover was added, the grid slid quickly under a binocular with enclosed substage lamp, and the reading made. The fish was then discarded. With a little practice reading could be done within 5 sec. of withdrawal from the background.

In determining the chromatic behaviour in total darkness it was found that for the operation of pouring on to the grid and covering the fish accurately, 2–3 sec. of very dim reflected light was desirable—and sufficient—in practice. A small area of bench between tank and microscope, fully screened from both, was used for this step.

Tests showed that this momentary departure from absolute darkness condition had no appreciable effect on the melanophore readings.

Readings were made with a binocular microscope with enclosed substage lamp as described for *Lebistes*.

All readings of *Salmo* were made at the site *X*, Fig. 14.

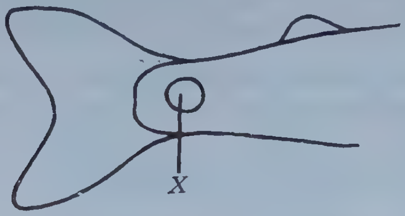

Fig. 14. *Salmo*. *X*=site of readings.

The time graphs shown in Figs. 15–17 are therefore constructed on the sam principle as those of Fig. 12 above, i.e. each point of the curve represents th mean melanophore index of six fish used for that point only.

(2) *Chromatic behaviour*

Mean equilibrium values	Melanophore index
On white background in light	1·5
On black background in light	4·4
In total darkness	1·2

The primary response is moderate, the range being approximately 0·5. Eyeles fish kept under standard lighting showed the following figures:

		Surviving					
° C.	No.	24 hr.	48 hr.	96 hr.	1 week	3 weeks	6 weeks
10–14	20	11	4	4	4	4	2
Mean melanophore index		3·1	3·0	2·8	2·1	2·0	1·75

The background responses in light of *Salmo* are shown in Figs. 15 and 16.

The response of *Salmo* transferred from black background to white backgroun in light is rapid, like that of *Lebistes*. Slowing down, however, occurs earlier, s that attainment of equilibrium at $\mu=1{\cdot}5$ takes 30 min. The response of *Salm* transferred from white to black background in light, however, is relatively slow and requires 10 hr. for completion.

On transference to black and white background in light after 7 days in tota darkness response to white background is complete within 1 hr. Equilibrium o black background is reached in 30 hr.

The chromatic behaviour of *Salmo* in darkness (Fig. 17), like that of *Lebistes* shows a time contrast to its behaviour in light. Fish transferred from white background in light to total darkness require 24 hr., and fish transferred from black background require 72 hr. to reach equilibrium at 1·2. The very low melanophore index of darkness equilibrium is significant, since it denotes a condition of pallor indistinguishable from that which occurs in fish placed on a white background in light.

VII. NATURE OF THE MECHANISM IN *LEBISTES* AND *SALMO*

The chromatic behaviour of *Lebistes* and *Salmo* presents striking contrasts with that of the eel. If the argument concerning the significance of time put forward at the beginning of this paper be accepted, the chromatic behaviour of the eel is clearly controlled by a humoral mechanism, while the existence of nervous co-ordination in *Salmo* and *Lebistes* is equally clear.

Salmo and *Lebistes* therefore appear to conform to the view that has been generally held hitherto concerning the nature of the co-ordinating mechanism in teleostean fishes.

Nervous control of melanophores in *Lebistes* and *Salmo* may readily be confirmed by weak electrical stimulation of the dorsal skin behind the head in animals equilibrated on a black background in light. Observed times of melanophore movement in animals so equilibrated, and hooded (with wet black paper) during the observation, were as follows:

	Melanophore movement	Mean time sec.	Recovery to initial state sec.
In the intact animal:			
Lebistes	4·0 → 1·5	32 ± 5	73 ± 7
Salmo	4·0 → 1·5	45 ± 3	107 ± 22
In the eviscerated animal:			
Lebistes	4·0 → 1·5	32 ± 9	98 ± 13
Salmo	4·0 → 2·0	49 ± 5	135 ± 11

With these reaction times in mind, further analysis of the mechanism of chromatic behaviour of *Lebistes* and *Salmo* may be essayed.

(*a*) *Behaviour in light*

Scrutiny of the time graphs of Fig. 10 shows that the response of *Lebistes*, transferred from a black background to a white background in light and vice versa, while rapid in its early stages subsequently slows down, so that the later phases of equilibration are distinctly more protracted than the earlier. This slowing down, more conspicuous in response to black background, suggests the possibility of a humoral element being involved also in co-ordination, and that the mechanism of the chromatic behaviour of *Lebistes* in light though predominantly nervous is reinforced by reflex hormonal control.

The same slowing down towards completion occurs in the response of *Salmo* to white background in light (Fig. 15). In *Salmo*, however, the response of animals transferred from a white background to a black background is definitely prolonged

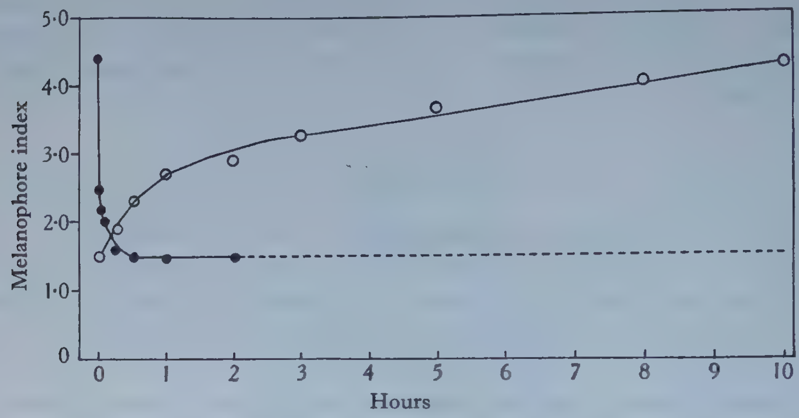

Fig. 15. *Salmo*. Response to black and to white background in light. Each point on the curves represents a separate group of six animals. Temp. 13·5° C. Lighting, 40 W. opal bulb at 24 in.

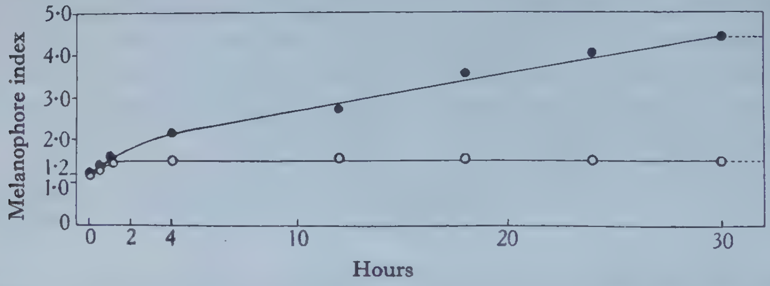

Fig. 16. *Salmo*. Response to black and white background in light of animals kept for 7 days in total darkness. Each point on the curves represents a separate group of six animals. Temp. 13·5° C. Lighting, 40 W. opal bulb at 24 in.

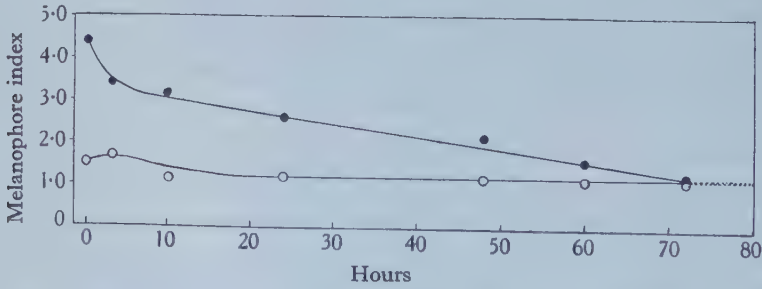

Fig. 17. *Salmo*. Response on transference from black and white background in light to total darkness. Each point on the curves represents a separate group of six animals. Temp. 13·5° C.

(10 hr.) in comparison with the white background response ($\frac{1}{2}$ hr.). *Ex hypothesi* it would appear to be co-ordinated less by nervous than by hormonal control. Control of chromatic behaviour of *Salmo* in light cannot then be described as wholly nervous.

This interpretation of the time relations of the chromatic behaviour of *Lebistes* and *Salmo* is consistent with the view that the control of the chromatic function n Vertebrates has evolved from a wholly humoral into a wholly nervous mechanism as in some reptiles), and that both mechanisms should coexist in some forms, whether both be functionally significant or otherwise. The existence among teleostean fishes of two sharply contrasting modes of chromatic behaviour in light, is seen in the eel and in *Lebistes*, suggests that in this group intermediate conditions may be expected.

(*b*) *Behaviour in darkness*

The time relations of the chromatic behaviour, and the reactions of the melanophores themselves, of *Lebistes* and *Salmo*, *in darkness*, are not consistent with wholly nervous co-ordination in either species.

A wholly nervous mechanism activated by light is inadequate to account for the following facts:

(1) Pale *Lebistes* (1·5) transferred to darkness from white background in light darken only slightly to 2·1, require 24 hr. for the change, and remain at that figure (Fig. 12).

(2) Pale *Salmo* (1·5) similarly transferred to darkness from white background n light continue to pale to 1·2, requiring 24 hr. for the change. Absence, in darkness, of the primary response, *might* account for the lowered figure, but the time needed is significant (Fig. 17).

(3) Dark *Lebistes* (4·2) transferred from black background in light to total darkness eventually pale to 2·1, and remain at that figure. They take 84 hr. to reach it (Fig. 12).

(4) Dark *Salmo* (4·4) transferred from black background in light into darkness fall to 1·2—a lower figure than in light on a white background—taking 72 hr. (Fig. 17).

There are thus two sets of facts which indicate that co-ordination in both *Lebistes* and *Salmo* is not wholly nervous.

(1) The prolonged time required in each case to reach equilibrium in darkness as compared with that required in light. These time intervals are plainly out with the limits of a nervously controlled mechanism. On the other hand, following what has been said in relation to the chromatic behaviour of the eel, the duration of the interval ${}_bT_d$ in both species is consistent with humoral control being nvolved.

(2) The state of the melanophores (melanophore index) arrived at *in the absence of light*.

To retrace the argument put forward in regard to the chromatic behaviour of the eel (p. 85, supra), if chromatic response to photic stimulation were determined entirely by the amount of light which falls on the retina, i.e. were solely nervous reflex, t might be expected that an animal kept in total darkness would reach a higher melanophore index than an animal kept on a black background in light; certainly not a *lower* melanophore index, when appropriately corrected for the primary reaction. This is not so, notably in *Salmo*, which is as pale in darkness as in ligh on a white background.

Thus the change from melanophore expansion to melanophore contraction, co vice versa, is not a single physiological process.

It is necessary therefore to distinguish between two distinct physiologica processes in examining chromatic response to photic stimulation: to distinguis between

(i) what happens when an animal is brought from light into darkness, an vice versa; and

(ii) what happens when an animal kept in light on a white background i transferred to a black background in light, or vice versa.

(*c*) *Co-ordination*

To say that co-ordination of chromatic behaviour in *Lebistes* or in *Salmo* is no wholly nervous might therefore mean one of two things:

(*a*) that one of the two processes referred to above is mainly controlled b peripheral innervation and the other is mainly controlled by internal secretion;

(*b*) that humoral control reinforces nervous control of one or both of them.

The data summarized in this section indicate that we may now draw a distinction between *Lebistes* and *Salmo* from this standpoint. In *Lebistes* the firs process is mainly, if not wholly, humoral, and the second is predominantly nervous In *Salmo* the first process is mainly, if not wholly, humoral, and the second provide clear evidence for the coexistence of both humoral and nervous control, th latter reinforcing the former.

VIII. CONCLUSION

Examination of the time relations of the chromatic behaviour of *Anguill vulgaris* furnishes evidence of the existence—hitherto unrecorded—among teleostea fishes of an exceedingly slow type of chromatic behaviour, co-ordinated by humoral mechanism. This contrasts with the rapid type of chromatic behaviou exhibited by other teleosts where nervous control is involved, as in *Lebistes* an *Salmo*.

Study of the time relations of the two latter forms, however, indicates tha co-ordination is not wholly nervous, especially in *Salmo* which provides clea evidence of the coexistence of both mechanisms, nervous and humoral.

These conclusions support the view which deems the evolutionary trend o the control of chromatic behaviour in vertebrates to have been from an archai humoral mechanism, known to be present in cyclostomes, elasmobranchs an Amphibia, from which, through the superimposition of a nervous mechanism, th exclusively nervous control exhibited by some reptiles has been reached.

Acknowledgement is made to the Carnegie Trustees for a grant towards th costs of the present investigation.

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A NERVE-MUSCLE PREPARATION FROM THE SNAIL

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(With Twelve Text-figures)

I. INTRODUCTION

In contrast to the Vertebrata, the Invertebrata as a whole do not lend themselves to the study of nerve-muscle preparations which can be isolated from the animal. The essential properties of a good nerve-muscle preparation are:

(1) That it should be possible to prepare the muscle free from other tissues and with its ends normally connected to relatively inextensible structures, e.g. bone or tendon, to which writing levers may be attached; and

(2) that it should be possible to prepare the nerve free from other tissues and that the nerve fibres in the nerve trunk should run direct to the neuro-muscular junctions without any other synaptic junctions intervening.

A nerve muscle preparation can be obtained from the snail (*Helix pomatia*) which, it is believed, very largely satisfies these conditions.

The muscle in question is the retractor of the buccal mass, a slip of the columellar muscle running from the columella to the ventral surface of the buccal mass. It varies in length from about 0·5 to 5·0 cm. according to the state of contraction. This slip is really double and is supplied by two nerves arising from the suboesophageal ganglion and entering the substance of the muscle near the buccal mass end. The anatomical arrangement is indicated in Fig. 1, and in a reasonably large snail it is possible to obtain a nerve trunk of about 1·0 cm. in length without undue stretching. Attachment can be made to the muscle without injuring it by making use of the buccal mass at one end and a small piece of the columella at the other.

The mechanical properties and heat production of this muscle have been studied by Bozler (1930*a*, *b*; 1931) and a certain amount of histological work has been done upon it by Plenk (1924). No work appears to have been done upon the nerve trunks supplying it, but the intra-muscular nerves have been described by Röchling (1922). In anticipation it may be mentioned here that the muscle responds to acetylcholine, and that neuro-muscular conduction is modified by such drugs as atropine, eserine and veratrine. In view of the recent advances in the humoral theory of neuro-muscular conduction which have been made in studies of Vertebrate physiology, his nerve-muscle preparation from the snail appears to afford an opportunity of eeing how far the principles applicable to the vertebrate system apply also in the Mollusca.

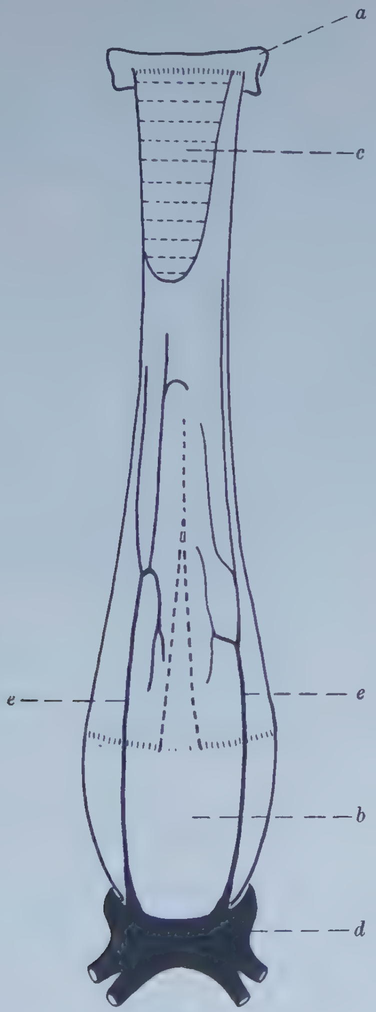

Fig. 1. *a*, columella; *b*, buccal mass; *c*, cut end of main columellar muscle; *d*, central nervous system; *e*,*e*., nerves to retractor of buccal mass.

II. METHOD OF PREPARATION

As already stated, this muscle has been used isolated from the snail's body by Bozler (1930*a*) and in that paper he has described a method of its preparation. Since, however, the nerves were not prepared by Bozler, it is thought advisable to give an account of the method used in the present work.

The snail is first bled by cutting through the first whorl of the shell just above its opening and by puncturing the wall of the haemocoele. After the blood ha drained out—usually about 4·0 c.c. are obtainable—the rest of the shell is cu away, leaving only that portion of the columella to which the muscles are attache The snail is then pinned out and the dorsal body wall is opened by a slit from th visceral mass to the head. The viscera are removed as far as possible. The nerv collar is slipped forwards over the buccal mass (if it is not already in this positio and the buccal mass is lifted with a pair of forceps: this serves to separate the re tractor from the rest of the columellar muscle and the main part of the muscle is cu through as near to the columella as possible. The small piece of columella with th muscle attached is then turned forwards.

The nerves are now exposed, as shown in Fig. 1. They are very thin an difficult to see except where they deform the surface film of moisture. In order t obtain as great a length of nerve as possible, the nerve collar is cut through on eac side just dorsal to the origins of the nerves: this enables ligatures to be tied nearer th origins than would otherwise be the case. As ligatures, fine strands of thread ar used, prepared by unravelling ordinary cotton thread into six strands. Manipulating these strands with fine forceps, one ties the ligatures and cuts away th surplus thread leaving only a piece about 1 cm. in length attached to each nerve Each of these threads is held in turn in the forceps and the nerves are cut between the ligatures and the nerve collar: they are then laid forwards over the muscle.

An ordinary thread ligature is tied round the buccal mass anterior to th attachment of the muscle and another is tied to the columella. The preparation ca then be cut away from the rest of the body.

Bozler reported that he was unable to find an artificial medium in which th preparation would behave satisfactorily and he was driven to use snails' blood to moisten the preparation. The same procedure has been followed in the presen experiments.

III. APPARATUS

Various methods of mounting this muscle have been used from time to time but for the present purpose it will be sufficient to describe two of these. The first A, was used when it was desired to record the mechanical response only: the second B, was used when electrical records were also required.

A. The arrangement is indicated in Fig. 2. The actual muscle chamber, *a*, wa a small ebonite trough lined with wax, 6 cm. long by 1 cm. wide and of depth varying between 0·5 and 1·0 cm. The muscle was set in this trough and attached t it by the thread of the buccal mass ligature which was waxed on to the end of th trough. The buccal mass was further secured by a pin passing through it and int the ebonite. The thread from the columella passed through a groove at the other en of the trough and was attached to the writing lever.

The "base", *b*, of the apparatus was a piece of ½ in. ebonite, with a slot int which the trough could be slipped and in which it was held by a clamping screw Into the base was fitted a brass tube, *c*, and upon this tube was mounted th adjustable carriage, *d*, of the writing lever *e*. In Fig. 2 an isotonic lever is shown, but this could readily be replaced by one of the "isometric" type if required.

There were two pairs of silver wire electrodes, each pair mounted upon an adjustable arm, and they could be made to dip into the trough. Only one such pair, *f*, is shown in Fig. 2. and the adjusting device is omitted for the sake of simplicity. The leads from the electrodes were brought to four terminals upon the ebonite base. About 2·5 c.c. of blood were required to cover the muscle, and the nerve, when laid over the electrodes was also completely immersed in blood.

The whole apparatus was mounted upon the vertical rod, *g*, of an adjustable stand and held in position by a tightening screw. The main advantage of the whole arrangement lay in the fact that the writing lever was carried upon the same support as the muscle, so that the whole system could readily be moved from one stand to another or could be raised and lowered without the necessity of having to adjust the connexion between the muscle and the lever.

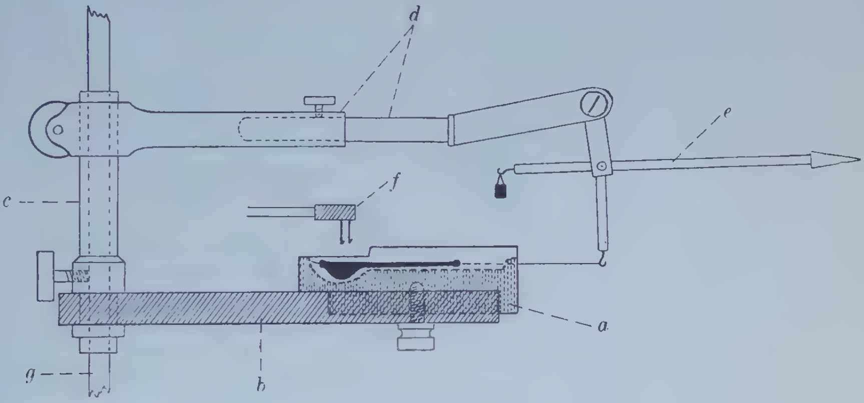

Fig. 2. For explanation see text.

B. For purposes of recording electrical changes it is not possible to have the preparation completely immersed in blood: and attempts to find a satisfactory moist-chamber technique, under which the nerve would remain viable for long periods, were unsuccessful. Apparatus B (Fig. 3) was developed to meet this difficulty, and its main feature was that a bath of blood, *a*, in which the preparation was normally immersed, could be lowered temporarily while a determination was being made. The buccal mass ligature was tied to a silver plate, *b*, which served as one of the recording electrodes. The columellar end of the muscle was impaled upon a silver hook attached to an isotonic lever, *c*, and this served as a second electrode. Besides these two electrodes, a silver pin, *d*, could be inserted into the muscle substance at any desired point: there were thus three possible leads from the muscle, *b*, *c* and *d*. The silver plate, and the isotonic lever were mounted upon a rod, *e*, with ebonite insulation (shown shaded in Fig. 3). The nerve was laid upon two pairs of fine gold or platinum electrodes, *f* and *g*, which could be used either for stimulating or for leading off. One of these pairs was carried upon the rod *e*; th other was carried upon an independent mounting.

The whole apparatus, excepting the upper part of the lever, was covered with a earthed gauze shield having a small slit sufficient to allow of the lever's movement

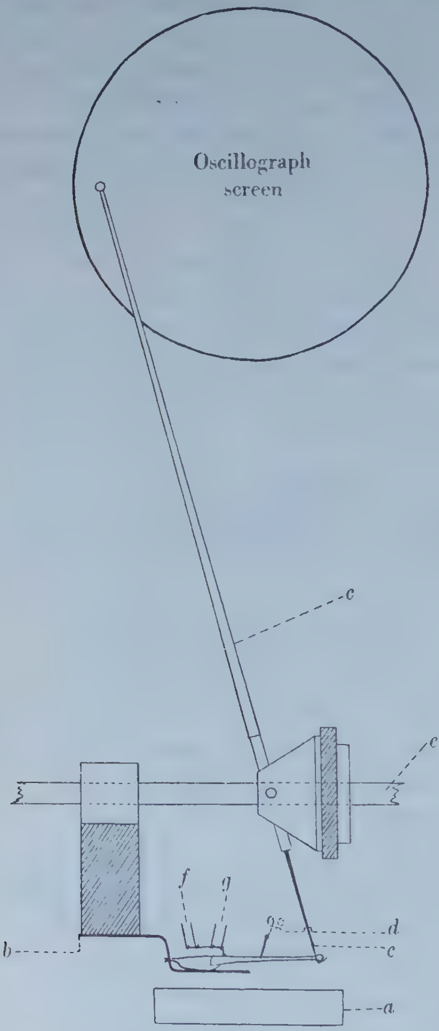

Fig. 3. For explanation see text.

The mechanical response could be recorded graphically or, alternatively optically. For graphical recording a writing point was attached to the top end of th lever and allowed to write upon a smoked drum with axis horizontal. For optica recording the writing point was replaced by a small gold bead. This was illuminate by a horizontal beam of light in the plane of movement and it reflected a point o light whose movements were recorded by a film camera. By arranging matters s that the gold bead traversed the screen of a cathode-ray oscillograph it was possible to obtain a record of mechanogram and electrogram simultaneously. Both muscle and nerve were led off to the oscillograph together and since the action current of the nerve was over before the action current of the muscle began, simultaneous records of the nerve action current, the muscle action current and the muscle contraction were obtained (see Fig. 9). This arrangement was not altogether satisfactory. The movement of the gold bead allowed for the complete contraction of the muscle to be recorded: but as the speed of the film was necessarily fairly rapid and as the mechanical responses to individual stimuli were a very small fraction of the total possible contraction, the mechanogram was not altogether easy to interpret.

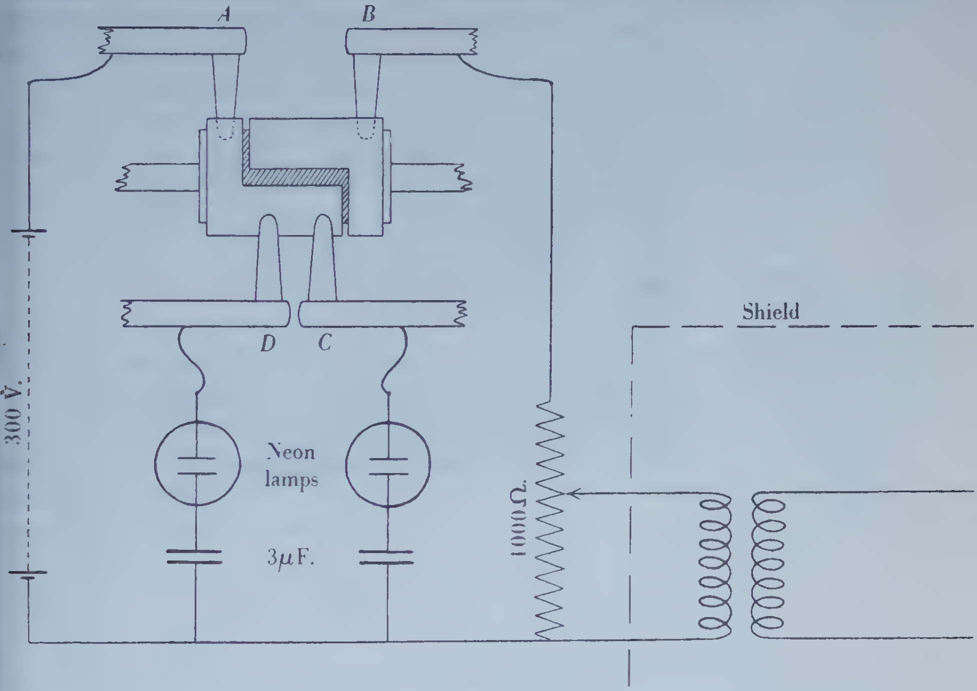

Fig. 4. For explanation see text.

The methods used for stimulating the preparation have been: alternating current (50 cyc./sec.): induction shocks: condenser discharges controlled by metronome, commutator or by automatic neon lamp or thyratron circuits. These methods are sufficiently well known to require no special description here, but an account is given below of two more special pieces of apparatus used in this work.

1. *The commutator*. This was simply a split ring upon which rested four phosphor bronze brushes (*A*, *B*, *C*, *D*, Fig. 4). One of these, *D*, was mounted upon an arm and could be rotated about the axis of the commutator so that the distance apart of *C* and *D*, measured over the circumference of the commutator, could be varied.

I am indebted to Dr W. A. H. Rushton for bringing to my notice the possibilities of this device, which can be put to various uses: in the present experiments it was used in connexion with the circuit shown in Fig. 4 to give two shocks at variable time intervals apart.

In order to prevent mains ripple being picked up by the stimulating circuit and carried to the oscillograph, a small transformer was interposed in the electrode leads after they passed within the shield of apparatus B.

2. *The multiple rheotome.* This instrument is shown in Fig. 5. It is built up upon a standard kymograph drum as supplied by Messrs Palmer. A $\frac{3}{4}$ in. steel rod, *a*, is screwed into the base and on this is carried a $\frac{1}{2}$ in. rod, *b*, free to rotate upon *a*. The rod *b* carries six knock-down keys of the type fitted to the Keith Lucas spring rheotome (Cambridge Instrument Co.). On the spindle, *c*, are carried six strikers. The spindle also carries a 360° protractor, *d*. A pointer, *e*, and a peep-sight, *f*, are carried on rod *a*. The pointer is sighted through the peep-sight and the zero of the protractor is brought into line with it: one of the strikers is then lined up with the pointer. The spindle is now turned until the required angle is indicated on the protractor and the next striker is lined up. In this way the strikers can be set at various angles and the time interval between the openings of any two keys will be proportional to the angle between the strikers and inversely to the speed of rotation of the spingle.

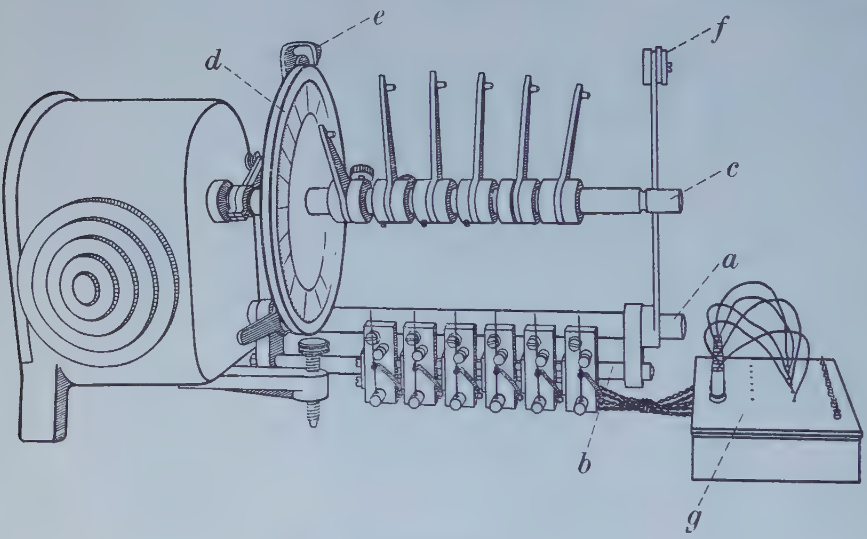

Fig. 5. For explanation see text.

This instrument has been used in conjunction with induction coils, but more satisfactory results have been obtained by the use of condenser discharges. Each key is interposed in the grid circuit of a thyratron valve and when this circuit is broken a condenser is allowed to discharge through the valve with a potentiometer in series.

The accuracy of this instrument is probably not high but it has been found sufficient for the present work.

I was fortunate in being able to make use of an apparatus built by Dr R. J. Pumphrey. It is expected that a description of this apparatus will appear shortly (Pumphrey *et al.* 1939) and it would not be proper for me to say more here than to indicate its capabilities. It consisted of an amplifier and cathode-ray oscillograph, the initial stages of the amplifier being direct-coupled or condenser-coupled according to the requirements of the experiment. It was also provided with a condenser-thyratron variable frequency stimulator and the sweep of the oscillograph could be automatically synchronized with each stimulus at all frequencies, so that the stimulus artefact and subsequent electrical response of the tissue could be viewed on the screen as a "stationary wave".

It is appropriate at this point to record my indebtedness to Dr Pumphrey. Not only did he spend much time explaining to me the use of the apparatus, but he also assisted me at many of the experiments and I have had the benefit of his advice upon many of the electrical and electro-physiological problems which have arisen in the course of this work.

IV. REPORT OF INVESTIGATIONS

Most of the snails used were obtained from France through the agency of Messrs Gaudin. Others were obtained from a chalk pit near Cambridge into which they were known to have been introduced some years ago. The stock was kept in a cold room—at about 5° C.—until required. About 48 hr. before the experiment the chosen specimens were brought into the laboratory and supplied with cabbage and water. It has been shown (Wells & Howes, 1934) that the snail's normal waking life exhibits a well-marked periodicity, and it was hoped that if this routine were adhered to, the animals would come to the experiment in approximately the same physiological state.

The preparation is very viable and has been known to respond to stimulation 72 hr. after removal from the snail.

Only one nerve (and therefore only one half of the muscle) was studied at any one time. In the earlier experiments the other half of the muscle was cut through, but, as this had no obvious effect upon the results, the practice was abandoned.

A. *The nerve*

As already mentioned, histological information about the nerve trunks supplying the retractor of the buccal mass is lacking. Other nerves in *Helix* have been studied, notably by Baecker (1932) who gives some account of their structure. It can readily be observed that a sheath consisting of fibrous tissue and "Blasenzellen" surrounds the nerve trunk proper, from which the latter can be separated. The nerve trunk is a very delicate structure, having the consistency of a jelly. It is impossible to tease it out and if compressed under a cover-slip it bursts, exuding a mass of apparently homogeneous protoplasm. According to Baecker, the shape of the nerve trunk i maintained by neuroglia fibres, forming a superficial membrane from which septa extend radially into the nerve trunk separating the nerve fibres into bundles o varying size. Still less information is available regarding the nerve fibres. Röchling

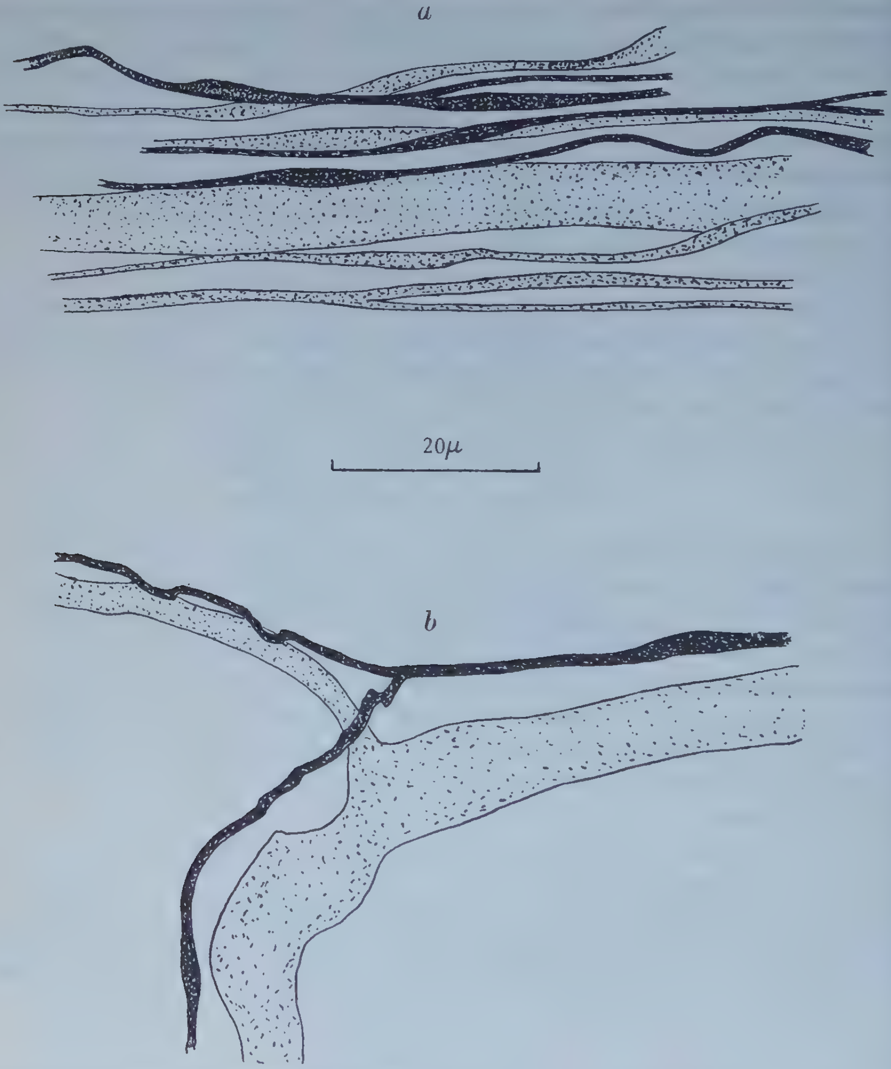

Fig. 6. *a*, camera lucida drawing of nerve fibres in nerve trunk; *b*, camera lucida drawing of intra-muscular nerve fibres.

(1922), using methylene blue, has noted that within the substance of the retractor muscle it is possible to distinguish two types of nerve fibre, one thick and lightly staining, the other thinner and more deeply staining, and that when branching occurs both types of fibre divided together. He compares this with the condition already known in the Crustacea.

A number of methylene blue preparations have been made of the nerve fibres, both in the nerve trunk and in the muscle. In the nerve trunk it appears that there are two types of nerve fibre present: the thicker, less deeply staining type is 6–10 μ in diameter, and the thinner more deeply staining type is 1–3 μ. The apparent diameter measured in optical section varies slightly from one part of the fibre to another. This is due partly to variation in the true diameter, and partly to variation in the disposition of fibres which are not truly circular in cross-section: partly also to the beaded appearance indicated in Fig. 6*a* which is almost certainly an artefact.

Branching of the thin fibres has been clearly observed in the nerve trunk and appearances highly suggestive of the origin of the thin fibres from the thick have been seen, but it has never been possible to follow fibres for a sufficient distance along the nerve to be absolutely certain that the thick fibres eventually break up into the thin ones. The greatest number of thick fibres observed in any nerve trunk was three: the number of thin fibres was often too great to count.

Within the muscle the two types of fibre have been identified and the method of branching commented upon by Röchling has been confirmed (Fig. 6*b*). It should be emphasized, however, that appearances of this type are by no means universal, and that when the fibres are followed through the muscle the distinction between the two types diminishes, and they no longer are distributed in parallel. The thick fibre becomes gradually thinner and indistinguishable from the thin one: eventually both types become vanishingly thin. No evidence has been found of differentiated motor nerve endings in these preparations, and no mention is made of them in the literature.

No evidence has been found of nerve cells in the course of the nerve fibres either in the nerve trunk or in the muscle.

The nerve action currents elicited by electrical stimulation present rather a complicated picture. Usually it is of the following type. Suppose that the nerve is subjected to a series of shocks at a frequency of 10 per sec. As the intensity of stimulation is raised to the threshold value a single impulse appears. When the intensity reaches about 1·5 × threshold a second impulse appears, arriving at the electrodes just after the first (Fig. 7*b*) and with still further increase a third impulse may appear after, and in addition to, the first two. But in some cases, as the intensity is increased, an extremely ragged volley is produced in which four or five impulses are suggested (Fig. 7*a*). The appearance of more than one impulse in response to a single shock may be attributed either:

(1) to the existence of nerve fibres with widely different conducting rates; or
(2) to repetitive discharges in some or all of the fibres; or
(3) to a combination of (1) and (2).

If a sufficient length of nerve were available it should be possible to decide whether the impulses are conducted at different rates or at the same rate: but it has not yet been possible to obtain measurements of any great accuracy. In one case when as great a length as 1·2 cm. of nerve was obtained, three pairs of electrodes were placed upon it. The pair nearest to the muscle were used as stimulating electrodes and the action current could be led off by either of the other two pairs. From the records it was quite clear that the further from the stimulating electrodes, the greater the distance between the first and second impulses on the record. This, however, might be expected with repetitive discharges also, if the second impulse was started during the relative refractory period following the first: but the following arguments can be put forward in addition to suggest that repetitive discharge is not the sole explanation:

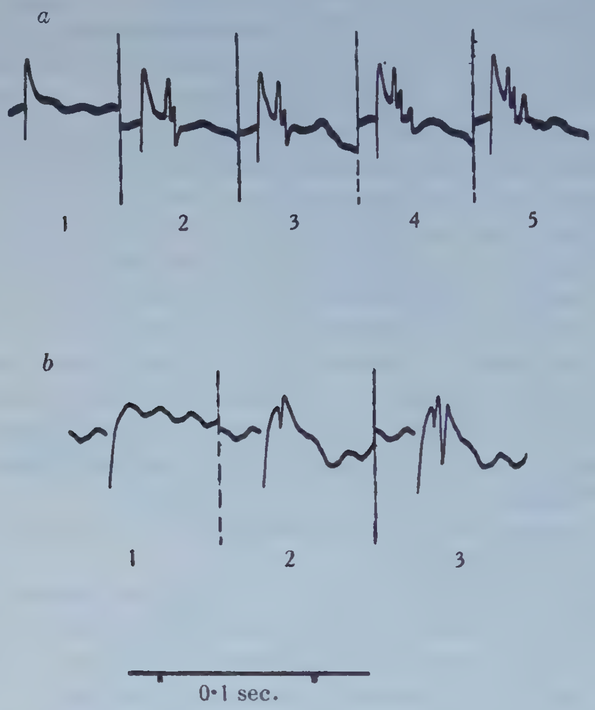

Fig. 7. *a*, upper record: leading-off electrodes at muscle end of nerve: 1, stimulus artefact; 2, first effective stimulus; 3–5, intensity increased to 3 × threshold. *b*, lower record: leading-off electrodes at central nervous system end of nerve (not the same nerve as above): 1, stimulus artefact; 2, first effective stimulus; 3, intensity approx. 1·5 × threshold. Condenser-coupled amplifier.

(1) As the intensity of stimulation is raised beyond the threshold of the second impulse, the amplitude of this impulse may exceed the amplitude of the first (Fig. 7*b*). It is improbable that the amplitude of a repetitive discharge will exceed that of the initial discharge in response to the stimulus.

(2) Records have been obtained in which, as the intensity is raised, the second impulse to appear on the record does so between the stimulus artefact and the first impulse: that is to say, it arrives at the leading off electrodes before the first impulse and therefore it cannot be a repetitive discharge in the same fibre or group of fibres.

(3) Not infrequently, as the intensity is raised to the threshold, two impulses appear *ab initio* (Fig. 7*a*).

(4) In view of the differences in the diameters of the fibres in the nerve trunk, differences in conduction rate are to be expected.

These facts suggest strongly that we are dealing here with nerve fibres of different conduction rates, but the possibility of repetitive discharge cannot definitely be excluded in all cases.

Since there is good reason to believe that we have two types of fibre in the nerve trunk, it is pertinent to enquire if these have different actions upon the muscle as has been shown for Crustacea (Marmont & Wiersma, 1938 and earlier publications therein referred to), for the cockroach (Pringle, 1939) and for the clam *Mya* (Pumphrey, 1938). It can only be stated, bluntly, that no physiological evidence even remotely suggestive of this conception has been obtained. Throughout most of the physiological work the histological differentiation of the nerve fibres was kept in mind, and any peculiarity of behaviour observed in the muscle was carefully studied from the point of view of double innervation. But in the end there was nothing to suggest that the response of the muscle, electrical or mechanical, was fundamentally different when the nerve carried two impulses or only one.

The following explanation is suggested as being in reasonable accordance with the facts as at present known. It is assumed that the nerve fibres leaving the central nervous system are of the thick type and that on their way to the muscle they divide up into fibres of the thin type: and that the level at which this division occurs varies from one fibre to another. It is not unjustifiable to assume further that the rate of conduction in the thick part of the fibre is more rapid than in the thinner branches. Thus if the nerve is stimulated at the central end and led off near the muscle, the impulse in a neurone which divides up near the muscle will reach the lead off before the impulse in a neurone which divides up early and so on. Further, this arrangement might account for an observation which can be made upon the results in general, namely, that, with the stimulating electrodes at the central end of the nerve and the leading off electrodes near the muscle, the nerve impulse volleys are more ragged than with the opposite arrangement.

If, then, the nerve trunk is composed of fibres differing widely in diameter and in physiological properties correlated with this, attributes of excitable tissues such as chronaxie, refractory period and rate of conduction cannot readily be defined except as maximum and/or minimum values.

Chronaxie. Lapicque (1935) has made determinations of chronaxie upon the nerve supply to the columella muscle of the snail. Contraction of the muscle was taken as index of nerve activity and single shocks were used. The value for chronaxie is given as 10 msec. and it is further claimed that the chronaxie of the muscle has normally the same value. When one remembers that in many invertebrate neuromuscular systems a single impulse in the nerve is not sufficient to cause visible contraction in the muscle, it is difficult to accept Lapicque's results without reservation. Attempts were made in the present work to determine the chronaxie of the nerves running to the retractor muscle: contraction of the muscle was taken as an index of nerve activity, but in this case series of shocks at various frequencies were used. It was found difficult to determine the rheobase satisfactorily: as the duration of the shock was increased, the minimal intensity sank slowly but steadily until the duration of each shock became of the same order as the interval between successive shocks. It was decided that the contraction of the muscle was not a suitable index of nerve activity for this purpose, and in the absence of an adequate moist chamber technique attempts to use the nerve action current instead were not made.

Refractory period. Five determinations were made of the refractory period of the nerve using the contraction of the muscle as index of activity. Two induction shocks, at varying intervals controlled by the multiple rheotome, were given and the intensity of the second shock could be varied. Strictly speaking, this method determines the refractory period of the neuro-muscular system as a whole, but the existence of a relative refractory period and its dependence upon the intensity of the second shock may be taken as showing that the refractory period of the nerve is the limiting factor.

Absolute refractory period (msec.)	Relative refractory period (msec.)
5·5	10
7	11
5·5	10
11	20
3	5·5

Rate of conduction. In view of the short length of nerve available and the slowness of the mechanical response of the muscle, no attempt was made to determine the rate of conduction by the classical technique of measuring the latent period of contraction. From the records of the experiment referred to on pp. 105–6, in which two pairs of leading off electrodes were laid upon the nerve, it is possible to estimate the conduction velocities with an accuracy of about ± 10%: they are 70 cm./sec. for the slow impulse and 140 cm./sec. for the fast impulse. These values compare with values obtained by Jenkins & Carlson (1903) for the pedal nerves of other Gasteropods:

Ariolimax columbianus	44 cm./sec.
Limax maximus	124 ,,
Pleurobranchaea californica	78 ,,

Mention might be made in passing of another finding by the same authors (Jenkins & Carlson, 1904), that the rate of conduction is hardly at all affected by stretching the nerve up to double its resting length.

B. *The muscle*

The histology of the muscle fibres of the snail has been described by Plenk (1924) according to whom the retractor of the buccal mass is composed of typical smooth muscle fibres, the total length of a stretched fibre being about 1 mm. Bozler (1930) reports that his attempts at maceration were not attended with great success, but that he succeeded in isolating fibres which in the stretched condition were equivalent to half the length of the muscle, thus suggesting that there are two sets of fibres arranged end to end. This suggestion is adumbrated by the general distribution of the nerves, whose bundles tend to break up at the two ends of the muscle, and by the following observation. If the last 0·5 cm. of the columellar end of a stretched muscle is killed by heat, it is noticed that stimulation of the nerve evokes a contraction of the buccal end of the muscle only: this contraction is observed as far as the middle of the muscle, and beyond the middle the columella end remains relaxed.

Notwithstanding the precautions described on p. 103 and that only well-extended, active animals were taken, the muscles were often in obviously different condition. When the dissection was performed some of the animals showed no more than a few transitory contractions of the musculature, and when the muscle was set up with a small weight (2 g.) it extended to its full length in a few seconds. But others would respond to the first cut with a violent and persistent retraction, and when set up the muscle might take 20 min. to relax fully. This may have been due to high viscosity, but with good mechanical magnification it could be seen that there were also very small spontaneous contractions, though it could not be decided with certainty in all cases that these did not arise from the musculature of the buccal mass. Generally, but not always, the muscles of the first type, i.e. those which relaxed readily, responded well to a single shock: in the second type the response to a single shock might be minute, but facilitation was more pronounced than in the first type (see later).

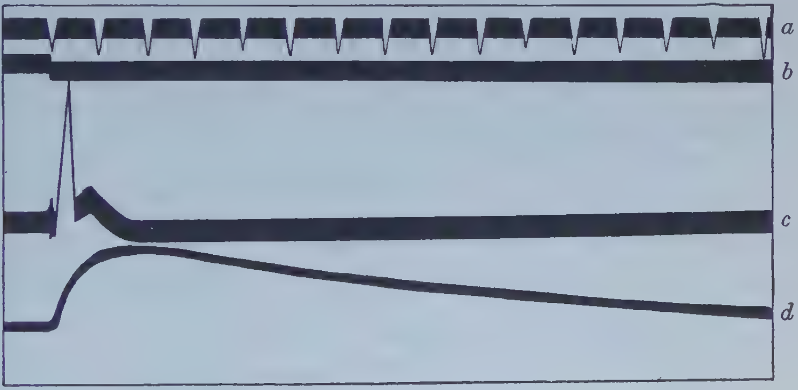

Fig. 8. From Bozler: *a*, time marker, 0·2 sec.; *b*, stimulus; *c*, electrical record; *d*, mechanical record.

Bozler (1930*a*) obtained simultaneous electrical and mechanical records from this muscle. He stimulated it directly and led off the two ends to a string galvanometer. Fig. 8 is taken from his paper and it is clearly the record from a muscle belonging to the first type: some of his records show no sign of facilitation at all. Bozler was mainly concerned with the mechanical properties of the muscle and he showed (Bozler, 1931) (1) that the form of the record of fall in tension after contraction ("erschlaffung") was the same as that for fall in tension after stretching ("relaxation"), and (2) that the rates of both these processes were greatly decreased by CO_2. In another communication (Bozler, 1930*b*) he studied the heat production and found $H/Tl = 0{\cdot}14$ as for frog's skeletal muscle. There is nothing abnormal in the mechanical properties of this muscle, and, in general, stimulation at a frequency of 10 per sec. will cause it to shorten completely against a load of 2 g.

The type of electrical record obtained from this muscle varies, as to some extent it must do, with the position of the electrodes. The most consistent results were

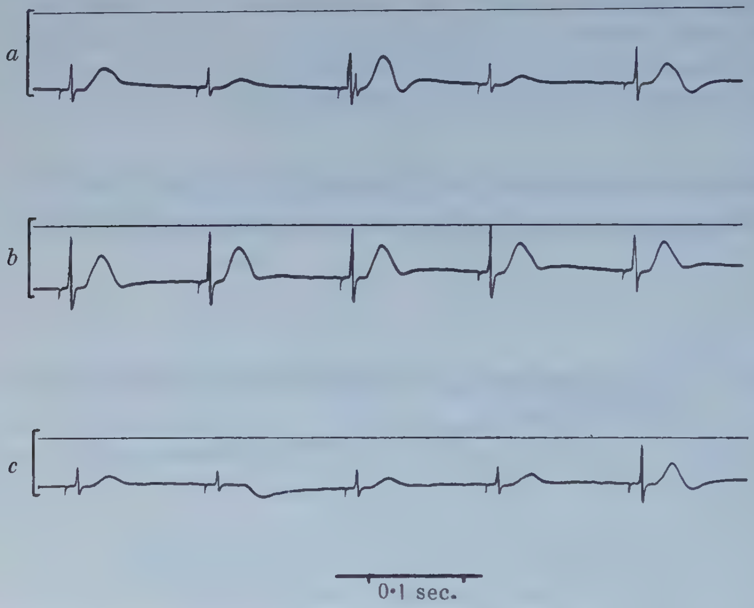

Fig. 9. The upper line shows the degree of shortening of the muscle: the lower line is the electrical record. Associated with each effective stimulus one can distinguish (1) the stimulus artefact, (2) the nerve action current (rapid, diphasic), (3) the muscle action current (slow, monophasic). These records are cuttings from a long series. Throughout *a* and up to stimulus no. 4 in *b* the intensity of stimulation was gradually increased up to 2 × threshold. Thereafter it was lowered to threshold—stimulus no. 2 in *c*—and increased again. Direct-coupled amplifier. A 1 Megohm variable resistance was used in series with the leads from the muscle and served to reduce the amplitude of its action current to the same order as that of the nerve. Electrodes *b* and *d* (Fig. 3), but columellar end of muscle not killed.

obtained in the following way. The columellar end of the muscle was killed with a jet of steam and the silver pin, *d* (Fig. 3), was stuck into the muscle rather on the buccal side of the half way line. The other electrode was the silver plate, *b*, which was in contact with the buccal mass. In this way almost completely monophasic records were obtained. If prolonged stimulation is to be applied to this muscle it must be allowed to shorten, otherwise it tears itself away from its attachments. The process of shortening necessarily alters the disposition of the electrodes and may lead to modification of the electrical record: this is not readily to be avoided.

Bozler's figure shows a very distinct spike followed by a slower potential change of lesser amplitude. Records of this type have been obtained in the present work also, but not consistently. The spike is always present, and may show indications of a double nature as in the latter part of records *a* and *b*, Fig. 10, or it may be followed by positive or negative after-potentials of varying duration. Even in the same preparation the form of the record is not consistent. Fig. 9 illustrates this very well: most records were very much more regular. Up to the present no correlation seems indicated between the precise form of the action current and the nature of the contraction.

It was mentioned above that neuro-muscular facilitation is evident in this preparation and that, like most of the other physiological properties, it is manifested in varying degree. In general, when two shocks are administered about 50 msec. apart the response to the two shocks is very much more than double the response to either alone. The relation of the interval between the shocks to the magnitude of the response has been studied from smoked drum records, but owing to the extremely small size of the contractions it was found more convenient to adopt the following modification of technique. Apparatus A was used with an "isometric" lever carrying a galvanometer mirror, and a beam of light was reflected from this on to a scale 5 ft. away. The excursions were read off the scale directly. The stimuli were condenser discharges controlled by thyratrons and the multiple rheotome, and the intensities of the two shocks were adjusted to give equal effects. The most consistent results were obtained with the following procedure. As zero, a point was chosen upon the scale which indicated a slight tension in the muscle: as the latter's

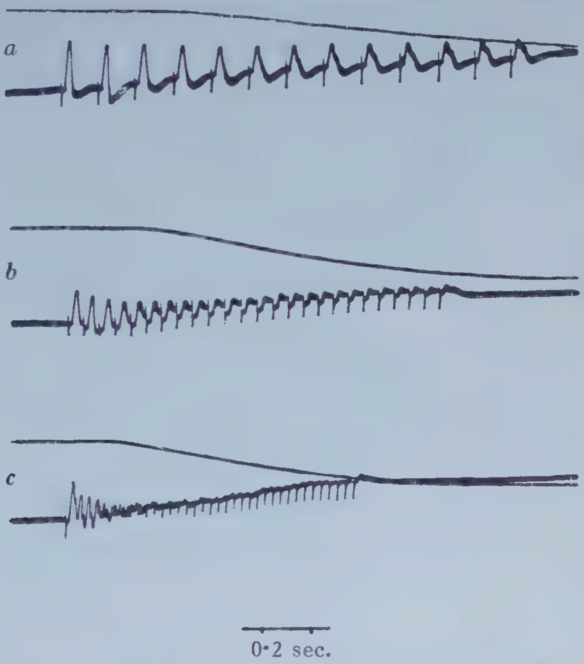

Fig. 10. Electrical and mechanical records from muscle only. Three different frequencies of stimulation, intensity 1·5 × threshold. Direct-coupled amplifier.

gradual relaxation from a previous contraction reached this value the next pair of stimuli was given. The intervals between successive contractions were of the order of 30 sec. A representative graph reproduced in Fig. 11 shows an optimum interval

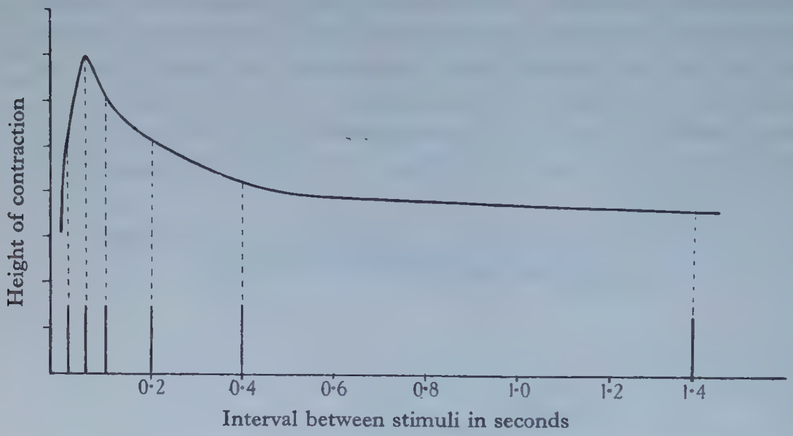

Fig. 11. The curve shows the height of contraction in response to two shocks at varying intervals. The height of contraction in response to single shocks is indicated by the heavy vertical lines.

of about 50 msec.: the refractory period is about 10 msec. and the facilitatory effect of the first stimulus is prolonged for well over a second. Other results suggest that where the response to a single shock is good the optimum interval is perhaps a little longer: the prolonged facilitatory effect is a feature of all the results so far obtained.

The electrical responses to two stimuli have also been studied. The muscle was mounted in apparatus B and the stimuli were given by the system illustrated in Fig. 4. Fig. 12 is a record of the effects of two stimuli given at increasing time-intervals apart. An interval of 11 msec. clearly falls inside the refractory period, but at 13 msec. a second spike appears and sums with the first to produce a peak not quite double the height of the single spike. As the interval is increased further and the second spike starts during the descending phase of the first, the total height reached becomes less and less and finally two separate and more or less identical spikes are shown.

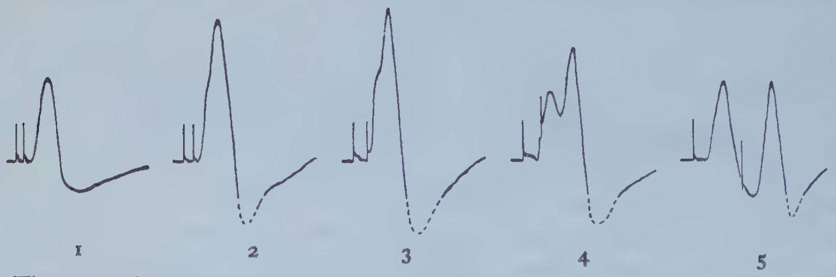

Fig. 12. These records are extracts from a series in which the time interval between two shocks was progressively varied. In these examples the time intervals were: (1) 11 msec.; (2) 13 msec.; (3) 24 msec.; (4) 39 msec.; (5) 90 msec. Intensity approx. 1·5 × threshold. Condenser-coupled amplifier.

This stage—at which the two spikes become identical in height—is reached when the interval between the stimuli approaches 100 msec. At shorter intervals it seems that some kind of summation does occur, but reference to Fig. 11 shows that the facilitatory effect of the first stimulus upon the contractile mechanism is prolonged over a much longer period than in the case of the action current mechanism, suggesting that these two mechanisms are not so closely associated as is often supposed. This suggestion is supported by other evidence: if a series of stimuli is given at high frequency the spikes rapidly dwindle in size, almost to extinction, while the contraction of the muscle is sustained more or less indefinitely (Fig. 10).

In another experiment the muscle was stimulated at a frequency of about 1 per sec. which gave normal action currents but a barely perceptible contraction. The frequency was increased to about 100 per sec. for half a second, and this reduced the action currents almost to nothing. The frequency was returned to 1 per sec. which was just sufficient to maintain the contraction which had developed in response to the higher frequency. Under these conditions the action current showed considerable recovery in about 2 sec.

No method has yet been devised whereby the muscle can be stimulated directly and the possibility of indirect stimulation via intramuscular nerves excluded.

V. DISCUSSION

It must be admitted that the results presented above are lacking in completeness and, in some cases, in precision also. But it is the object of this communication to present a brief survey of the behaviour of this nerve-muscle preparation and it was decided to publish the results in their present form before attempting further investigation of the various problems which have arisen and of which many may prove to be somewhat difficult. More especially this applies to the histological side where the question of double innervation cannot be considered as settled. Attempts to develop silver impregnation methods for studying the nerve fibres and their distribution failed completely and were abandoned. On the physiological side the effects of ions, drugs, etc. have barely been touched upon.

At first one might imagine that, of all the smooth muscles so far examined, the anterior retractor of the byssus of *Mytilus* (Fletcher, 1937*a*, *b*, *c*) would be the most similar in behaviour. This however, is not the case: there are several distinct differences in behaviour between the muscles of *Helix* and of *Mytilus* and not all these differences can be explained as due to differences in technique.

(1) In *Mytilus* the muscle fibres run the whole length of the muscle: in *Helix* probably not more than half-way.

(2) In *Mytilus* the motor nerve supply is as yet unknown and therefore there is no evidence of neuro-muscular facilitation as there is in *Helix*.

(3) With a series of stimuli at about 10 per sec. in *Helix* the muscle action currents rapidly decline: in *Mytilus* they show a staircase effect.

(4) In *Mytilus* alternating current and direct current elicit different mechanical responses: this does not appear to be the case in *Helix*.

The *Mytilus* muscle seems more comparable to the abductor of *Pecten* (Bozler 1930*a*) and perhaps to the adductor of Mya (Pumphrey, 1938) than to the *Heli* muscle.

On the other hand the electrical records of Fig. 10 show an obvious similarit to the records published by Rosenblueth *et al.* (1936) for the nictitating membran of the cat. Lambert & Rosenblueth (1935) in an earlier study have analysed th rather complex electrogram of this muscle, recognizing three components; I, an obvious spike, II, a slower and smaller potential change of much the same type closely following upon I, and III a very slow potential change occurring much later Eccles & Magladery (1937*a*, *b*) have also identified two potential changes, A and B which may be the same as Rosenblueth's I and II. In the latter part of records and *b*, Fig. 10, it is evident that the spike is of a double nature and that as stimulation proceeds the second component appears to increase at the expense of the first Bozler's records (Fig. 8) also show two components, but so far no evidence of third has been found in the snail. The interpretation of the electrogram of the cat's nictitating membrane is still controversial (see also Bacq & Monnier, 1934), and in view of the relative lack of information about the snail's muscle it would be unprofitable at this stage to discuss the findings herein reported in relation to the various theories advanced for the nictitating membrane.

Such investigation as has been made of the effects of ions and drugs upon this preparation is not worth reporting in detail. As mentioned in the introduction this muscle is sensitive to acetylcholine. In the presence of eserine (1/15,000) it will respond to a concentration of 1/1,500,000 acetylcholine with a powerful and prolonged contraction: without eserine it will respond to a concentration of 1/800,000 acetylcholine. Bacq (1937) reports contraction of the foot in *Buccinum* to 1/50,000 acetylcholine. In a later paper by Bacq & Coppée (1937) eserine (1/50,000), atropine (1/1000) and curare (1/1000) were found to be without effect upon the contraction of the foot of *Buccinum* in response to stimulation of the pedal nerves. In *Helix* atropine in concentrations of about 1/1000 does have some effect in depressing the response to the nerve and to acetylcholine, but this is admittedly a high concentration. Crude curare is without demonstrable effect. Eserine in moderate concentrations (1/15,000) weakens the response to the nerve: in high concentrations (1/3000) it evokes spontaneous contractions of the muscle. Veratrine (about 1/300,000) causes repeated action currents and a sustained contraction in response to a single shock applied to the nerve. Precipitation of calcium by sodium oxatate gives rise to irregular spontaneous activity which can be suppressed by the addition of calcium chloride. Potassium in slightly increased concentration potentiates the response to the nerve: as the concentration is further increased the response to the nerve weakens and then the muscle goes into a state of contraction spontaneously. It should be emphasized that the findings recorded in this paragraph are based on a very limited number of experiments and require confirmation.

It is proposed, next, to investigate more fully the role of acetylcholine in neuromuscular transmission and to extend the range of the facilitation experiments to more than two stimuli. One of the main objects in presenting the results in their present form is to draw attention to the possibilities of this preparation and it is hoped that some of its problems will be considered worthy of investigation by other workers.

VI. SUMMARY

1. A nerve-muscle preparation from the snail and its method of removal from the animal are described.
2. Further description is given of the apparatus used for obtaining records of its activity.
3. The nerve appears to contain fibres which fall into two groups according to their diameters. It is suggested that the thin fibres are derived from the thick ones.
4. No physiological evidence of double innervation has been found.
5. There is clear evidence of neuro-muscular facilitation.
6. The muscle contracts in response to low concentrations of acetylcholine.

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